CIBA FOUNDATION COLLOQUIA ON ENDOCRINOLOGY

Vol. VII. Synthesis and Metabolism of Adrenocortical Steroids

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VOLUME VII

Synthesis and Metabolism of Adrenocortical Steroids

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FOREWORD

by

G. F. Marrian, D.Sc., F.R.S.

Readers of this volume, even though they may be experts in the steroid field, can hardly fail to be deeply impressed—as I was while listening to the papers read at the colloquium—by the evidence of the amazingly rapid advance in our knowledge of the chemistry and biochemistry of the steroids during the past few years. From the early 1930's, when the chemical relationship between the gonadal hormones and the sterols first became apparent, to the late 1940's, the advances in our knowledge of these matters were by no means inconsiderable; but in view of the obvious biological importance of the steroid hormones and in view of the great resources of modern organic chemistry and biochemistry it seems surprising in retrospect that these advances were not more rapid during this period, and that steroid research was for so long widely regarded by both organic chemists and biochemists as a rather esoteric occupation.

Steroid research lost its esoteric character in 1949 almost overnight following the announcement from the Mayo Clinic of the dramatic effects of the administration of cortisone on the symptoms of rheumatoid arthritis. In the opinion of some clinicians the early claims made on behalf of cortisone as a therapeutic agent were over-optimistic, and it might therefore seem that the importance of the Mayo Clinic discovery may be less than was originally supposed. However, even in the highly unlikely event of the treatment of rheumatoid arthritis with cortisone becoming completely discredited, the Mayo Clinic discovery would still rank as one of the

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in the biogenesis of the steroid hormones—and in particular of the adrenocortical hormones; and we are beginning to learn something about the enzyme systems controlling these biosynthetic processes and the physiological inactivations of the secreted hormones in the tissues.

The task for the immediate future is to discover more about the chemical mechanisms involved when the steroid hormones produce their characteristic physiological actions on their target organs. Now that the steroid biochemists have become actively interested in enzymes and the enzyme biochemists are aware of the importance of certain of the steroid hormones in enzymically controlled metabolic processes, we may expect that within the next few years we should gain some understanding of this fundamentally important problem. The determinations at this conference may well hasten its solution.

greatest in modern medical research because of the gre stimulus it provided for chemical and biochemical work c the steroids. As a direct and immediate result of this di covery the tempo of work in laboratories already engaged steroid research was greatly accelerated, while many ab chemists and biochemists became attracted to it from oth fields.

For several years now there has been no lack of highl skilled organic chemists willing and eager to tackle th formidable problems involved in the synthesis and pseudo synthesis of the steroid hormones. Greatly improved methoc have been developed for the economical production of variou adrenocortical hormones from different animal and plan steroids and sterols, and so efficient are certain of these, tha cortisone, which until recently was a rare and costly drug, i now available in plentiful supply at a reasonable cost. Durin the past few years we have even seen the development o methods for the total synthesis of many of the non-aromati steroids and sterols—achievements which must surely ranl as amongst the greatest of the synthetic organic chemists There is no doubt that steroid chemistry has, in the course o a few years, grown to become one of the major branches o organic chemistry.

To me as a biochemist, the recent developments in steroic biochemistry are even more gratifying than those in the "pure" chemistry of the steroids. Partly as a result of the stimulus of the cortisone discovery and partly in consequence of the availability of solvent-partition chromatographic methods, progress in the important tasks of isolating and identifying steroid hormone metabolites in urine and blood and in developing methods for their quantitative determination has been enormously accelerated. Important though this progress undoubtedly is, it must yield first place to the developments in the newer steroid biochemistry, which is concerned with the biosynthesis, metabolism and mode of action of the steroid hormones. In recent years we have begun to obtain some insight into the chemical mechanisms involved

NOMENCLATURE

The nomenclature of steroids in this book is based on the recommendations of the Ciba Foundation Conference in June 1950. These suggestions have been widely adopted (*J. chem. Soc.*, 1951, p. 3526; *Helv. chim. Acta*, 1951, **34**, 1680; *Bull. Soc. chim. Fr.*, 1951, p. i). The systematic names have been used almost exclusively in the more chemical papers, but in the latter half of the Symposium, which consists of more biological papers, many trivial names have been used.

It seems desirable to have in one place in this book a list of all the known adrenal steroids and of the principal related steroids which are discussed here. The table, given on pp. x and xi, is based on the well-known table of Reichstein and Shoppee (*Vitamins and Hormones*, 1943, **1**, 352); it includes some of the many trivial names which have been introduced, particularly in the last few years since the introduction of cortisone into clinical work. The table includes the alphabetical designations given to the adrenal steroids by the three principal schools of Reichstein, Kendall and Wintersteiner. In spite of all that may be said—even by those who discovered the compounds (cf. Professor Reichstein's remarks on p. 229 of this volume)—it seems probable that these convenient alphabetical designations will continue to be used for many purposes. It is essential, however, that whenever they are used, the name of the author should be included (e.g. Reichstein's S, Kendall's E), and that the systematic name be given at the first mention in any paper.

W. Klyne

No.	Empirical formulae	Systematic name (and trivial name, if any)	Alphabetical designation: T. Reichstein	Kendall	Wintersteiner
13	$C_{21}H_{32}O_4$	5α-Pregnane-3β 21-diol-11 20-dione	N	H	—
14	$C_{21}H_{30}O_4$	Pregn-4-ene-20β 21-diol-3.11-dione	T	—	—
15	$C_{21}H_{30}O_4$	Pregn-4-ene-11β 21-diol-3 20-dione (Corticosterone)	H	B	—
16	$C_{21}H_{28}O_4$	Pregn-4-en-21-ol-3 11.20-trione (11-Dehydrocorticosterone)	—	A	—
17	$C_{21}H_{28-30}O_4$	αβ-Unsaturated ketone: (constitution unknown)	—	—	—
		$C_{21}O_3$ group			
18	$C_{21}H_{36}O_3$	5α-Pregnane-3β 17α·20β-triol	J	—	—
19	$C_{21}H_{36}O_3$	5α-Pregnane-3β:17α 20α-triol	O	—	—
20	$C_{21}H_{34}O_3$	5α-Pregnane-3β 17α:diol-20-one	L	—	G
21	$C_{21}H_{30}O_3$	Pregn-4-en-17α-ol-3 20-dione (17α-Hydroxyprogesterone)	—	—	—
22	$C_{21}H_{30}O_3$	Pregn-4-en-21-ol-3 20-dione (11-Deoxycorticosterone; DOC ‡)	Q	—	—
		$C_{21}O_2$ group			
23	$C_{21}H_{34}O_2$	5α-Pregnan-3β-ol-20-one	—	—	—
24	$C_{21}H_{30}O_2$	Pregn-4-ene-3 20-dione (Progesterone)	—	—	—
		C_{19} group			
25	$C_{19}H_{30}O_3$	Androstane-3β 11β-diol-17-one	—	—	—
26	$C_{19}H_{24}O_3$	Androst-4-ene-3 11 17-trione (Adrenosterone)	—	—	—
27	$C_{19}H_{26}O_2$	Androst-4-ene-3 17-dione	—	—	—
		C_{18} compound			
28	$C_{18}H_{24}O_2$	Œstrone	—	—	—

Some related steroids for which trivial names are used in the text.

29	$C_{21}H_{34}O_5$	5β-Pregnane-3α 11β 17α 21-tetrol-20-one (Tetrahydro-F)
30	$C_{21}H_{32}O_5$	5β-Pregnane-3α·17α 21-triol-11 20-dione (Tetrahydro-E, tetrahydrocortisone)

The 5α-pregnane or *allo*pregnane isomers are sometimes called '*allo*tetrahydro-F and -E'

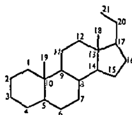
31	$C_{21}H_{30}O_5$	5β-Pregnane-17α 21-diol-3 11 20-trione (Dihydro-E, dihydrocortisone)

The 11-epimers of compounds with trivial names (11α-hydroxy compounds) are often referred to as '11-*epi* F', '11-*epi*-hydrocortisone', etc

‡Acetate commonly called 'DOCA'; 'DCA'.

STEROIDS FROM THE ADRENAL GLAND AND RELATED STEROIDS

Steroids isolated from the adrenal gland (Based on the table published by Reichstein and Shoppee (1943) *Vitamins and Hormones*, **1**, 352).



No.	Empirical formulae	Systematic name (and trivial names, if any)	Reichstein	Kendall	Wintersteiner
		$C_{21}O_5$ group			
1	$C_{21}H_{36}O_5$	[illegible]	A	D	A
2	$C_{21}H_{34}O_5$	[illegible]	V	—	—
3	$C_{21}H_{34}O_5$	[illegible]	C	C	D
4	$C_{21}H_{32}O_5$	[illegible]	D	G	?B
5	$C_{21}H_{32}O_5$	[illegible]	E	—	—
6	$C_{21}H_{30}O_5$	[illegible]	U	—	—
7	$C_{21}H_{30}O_5$	[illegible] cortisone†)	M	F	—
8	$C_{21}H_{28}O_5$	Pregn-4-ene-17a 21-diol-3 11 20-trione (17a-Hydroxydehydrocorticosterone; cortisone)	Fa	E	F
		$C_{21}O_4$ group			
9	$C_{21}H_{36}O_4$	5a-Pregnane-3β 17a 20β 21-tetrol	K	—	—
10	$C_{21}H_{34}O_4$	5a-Pregnane-3β 17a 21-triol-20-one	P	—	—
11	$C_{21}H_{30}O_4$	Pregn-4-ene-17a 21-diol-3 20-dione	S	—	—
12	$C_{21}H_{34}O_4$	5a-Pregnane-3β.11β 21-triol-20-one	R	—	—

*5a-Pregnane is also commonly called '*allo*pregnane'

†Prof C. W. Shoppee has pointed out that 'cortisol' would be a much better trivial name for this compound. 'Hydrocortisone' invites confusion with 'dihydrocortisone' (see No. 31)

CONTENTS

List of those participating in or attending the Colloquium on The Synthesis and Metabolism of Adrenal Cortical Steroids 7th–10th July, 1952

C. R. Addinall	Merck & Co., Inc., Rahway, New Jersey.
G. Anner	Ciba Ltd., Basle.
D. H. R. Barton	Dept. of Chemistry, Birkbeck College, London.
R. Borth	Clin. Univ. de Gyn et Obstét., Geneva.
I. E. Bush	National Institute for Medical Research, London.
R. K. Callow	National Institute for Medical Research, London.
H. M. E. Cardwell	Dyson Perrins Lab., Univ. of Oxford.
J. W. Cook	Dept. of Chemistry, Univ. of Glasgow
C. Djerassi	Dept of Chemistry, Wayne Univ., Detroit.
R. I. Dorfman	Worcester Foundation for Experimental Biology, Shrewsbury, Mass.
L. F. Fieser	Dept. of Chemistry, Harvard Univ
Mary Fieser	Dept. of Chemistry, Harvard Univ.
M. Finkelstein	Rothschild Hadassah Univ. Hospital, Jerusalem.
S. J. Folley	National Institute for Research in Dairying, Reading.
C. H. Gray	Dept. of Chemical Pathology, King's College Hospital, London.
O. Hechter	Worcester Foundation for Experimental Biology, Shrewsbury, Mass.
B. A. Hems	Glaxo Laboratories, Greenford, Middx.
H. Heusser	Eidg. Technische Hochschule, Zürich
H. Hirschmann	Dept. of Medicine, Western Reserve, Univ. of Cleveland, Ohio.
Frieda Hirschmann	Dept. of Medicine, Western Reserve, Univ. of Cleveland, Ohio.
E. R. H. Jones	Dept of Chemistry, Univ of Manchester.
F. W. Kahnt	Ciba Ltd., Basle
W. Klyne	Postgraduate Medical School of London
G. F. Marrian	Dept. of Biochemistry, Univ. of Edinburgh.
C. J. O. R. Morris	Endocrine Unit, The London Hospital, London

PART I

SYNTHESIS OF ADRENOCORTICAL AND RELATED STEROIDS

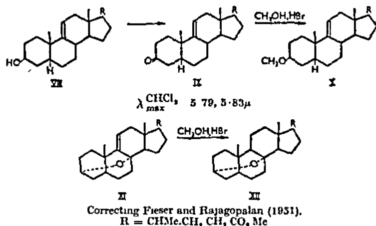
COMPANION SUBSTANCES IN CHOLESTEROL OF VARIOUS SOURCES

LOUIS F. FIESER

THE programme of the colloquium includes several papers on the utilization of sterols for the synthesis of cortical hormones. Although I shall speak on another subject, it seems appropriate to present two comments regarding the work of my group on the synthesis of 11-ketosteroids. One is that, in the heat of the early competition and with the limitation of space allowable in Communications to the Editor, I have not previously had an opportunity to express an acknowledgment. Our first method for synthesis of an 11-ketosteroid starting with a Δ^5-stenol (Fig. 1) is assuredly less efficient than methods subsequently reported utilizing some of the same intermediates. The first step, introduction of the 7·8-double bond (II), is a transformation first accomplished by Windaus, Lettré and Schenck (1935) in the classical vitamin D work at Göttingen. Windaus also discovered methods for selective reduction of an ergosterol-type diene to a Δ^7-stenol (III) (Windaus and Brunken, 1928), and for dehydrogenation of the latter with mercuric acetate to a $\Delta^{7,9(11)}$-diene (IV) (Windaus and Auhagen, 1929). In an early experiment conducted before the structures were known, Windaus (1906) effected the first 1:4-oxygenation of a steroid diene: $\Delta^{3,5}$-cholestadiene on chromic acid oxidation afforded Δ^4-cholestene-3.6-dione.

A. S. PARKES	National Institute for Medical Research, London
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T REICHSTEIN	Organisch-chemische Anstalt der Universität, Basle.
MRS. A. M. ROBINSON	Cancer Research Unit, St. Bartholomew's Hospital, London.
SIR ROBERT ROBINSON	Dyson Perrins Lab., Univ of Oxford.
L T. SAMUELS	Dept. of Biological Chemistry, Univ. of Utah
L. H. SARETT	Merck & Co , Research Div., Rahway, N.J.
C W. SHOPPEE	Dept of Chemistry, Univ. College of Swansea.
SYLVIA A. SIMPSON	Courtauld Inst. of Biochemistry, Middlesex Hospital, London
I. F. SOMMERVILLE	Inst. of Obstet & Gynaec., Chelsea Hospital for Women, London
F. SONDHEIMER	Syntex S.A., Mexico.
F. S. SPRING	Dept. of Chemistry, Royal Technical College, Glasgow.
M. STACK-DUNNE	Dept. of Biochemistry, Univ of Cambridge.
J F. TAIT	Barnato Joel Laboratories, Middlesex Hospital, London
ELEANOR H. VENNING	Univ. Clinic, Royal Victoria Hospital, Montreal
J. WEISS	Dept. of Chemistry, King's College, Newcastle-upon-Tyne.
D. C. WILLIAMS	Endocrine Unit, The London Hospital, London
F. G. YOUNG	School of Biochemistry, Univ. of Cambridge.

Improvements in our Baird spectrophotometer have made possible resolution of the band at 5·8 μ into a doublet; the oxidation product is the 3-ketone (IX), evidenced by formation of a semicarbazone and a 2:4-dinitrophenylhydrazone. The product of hydrogenation in methanol containing hydrobromic acid does indeed contain an inert oxygen atom, but the substance is the methyl ether (X) and not an oxide. The β-orientation of the methoxyl group was established by preparation of an identical compound by methanolysis of



Correcting Fieser and Rajagopalan (1951).
R = $CHMe.CH_2CH_2CO_2Me$

FIG. 2.

methyl $\Delta^{9(11)}$-lithocholenate 3-tosylate. The novel reaction, which we propose to describe as a reductive methylation, has been applied also to methyl dehydrolithocholate, to coprostanone, and to cholestanone. (See Babcock and Fieser, 1952, published subsequently.)

On the occasion of the Conference on Steroid Nomenclature held at the Ciba Foundation in the summer of 1950, I became interested through conversations with Sir Ernest Kennaway and with Dr. I. Hieger in the possible existence of a non-aromatic steroid carcinogen derived from or related to cholesterol.

This example inspired the work in my laboratory on $\Delta^{7,9(11)}$-dienes of the cholesterol and bile acid series, and I recall conducting the first exploratory oxidation on the opening day of the fall term in September, 1950. Even the next to last step in the process, reduction of V to VI, follows the pattern mapped out by Windaus's reduction of Δ^{4}-cholestene-3.6-

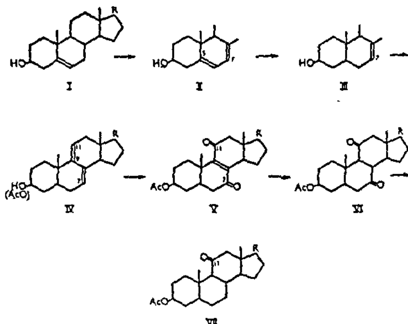


FIG. 1.

dione to cholestane-3:6-dione with zinc and acetic acid (Windaus, 1906). Hence the current work has derived enormous help and inspiration from the pioneer work of the great master, Adolf Windaus.

My second comment is with regard to a product of oxidation of methyl $\Delta^{9(11)}$-dehydrolithocholenate (Fig. 2) tentatively formulated by Fieser and Rajagopalan (1951) as a 8:8-oxide.

acid. The apology was repeated by Diels and Abderhalden (1903), who introduced the use of sodium hypobromite. The incentive, in these and other early studies of oxidation, was elucidation of structure, and substantial contributions to this general problem by the Windaus school resulted in isolation of volatile fragments from oxidative fission of sterol side chains: methyl *iso*hexyl ketone (Windaus and Resau, 1913), acetone (and a C_{22}-acid) (Windaus and Neukirchen, 1919), methyl and ethyl*iso*propylacetaldehyde (Reindel and Kipphan, 1932; Guiteras, 1932; 1933). Ruzicka's isolation of ketonic nuclear fragments of oxidative fission in 1934 paved the way for eventual production of all the principal sex hormones from sterols. Oxidation of cholesteryl acetate by the Göttingen group (Windaus, Lettré and Schenk, 1935) was the key step in the synthesis of provitamin D_3.

One incentive to undertake fresh work on a very old problem has been mentioned; another was interest in the mode of formation of three neutral products of chromic acid oxidation characterized by Mauthner and Suida (1896). A new method of attack arose from the observation that oxidations can be conducted smoothly with a solution of sodium dichromate dihydrate in anhydrous acetic acid and that on addition of one-half volume of benzene the reaction can be conducted at 0° in homogeneous solution. Mauthner and Suida's "oxycholestenediol" is now known to be cholestane-3.6-dion-5α-ol; it probably arises in part from cholesterol through the oxide and in part from cholestane-3β.5α 6β-triol, which has been isolated by my associate Dr. Bidyut Bhattacharyya from gallstone, egg yolk and brain cholesterol. Mauthner and Suida's "oxycholestenone" has long been characterized as Δ^4-cholestene-3·6-dione, but the structure of their "α-oxycholestenol" has remained uncertain. My experiments suggest that "α-oxycholestenol" (m.p. 180°) was a somewhat impure preparation of an oxidation product that when fully purified melts at 195° and is identical with the known 6β-hydroxy-Δ^4-cholesten-3-one (Ellis and Petrow, 1939) (Fig. 3, XVI). I have oxidized the substance to the

The available brief of evidence, summarized below, is far from definitive, but it seemed to me sufficiently suggestive to warrant initiation of chemical studies.

Question of a Steroid Carcinogen

I. Hieger (Royal Cancer Institute, 1949).

Cholesterol in lard solution injected into several hundred mice produced 4–6 per cent incidence of tumours at the site of injection.

F. Bischoff and J. J. Rupp (Santa Barbara Hospital, 1946).

Crude progesterone, made from cholesterol by bromination and oxidation, gave 32 per cent incidence of tumours at the site of injection in mice. Pure progesterone was negative.

A. H. Ruffo (1934), confirmed by *H. P. Rusch and C. A. Baumann* (1939).

Irradiation of mice increases incidence of skin cancer. Irradiated cholesterol evokes skin cancer in mice.

T. Leary (Boston, 1950).

". . . the evidence leaves little doubt that cholesterol is intimately related to certain human and experimental tumors".

A first hypothesis (Fieser and Schneider, 1952) that the supposed carcinogen is an abnormal cholesteryl ester, for example the *iso*heptylate, formed from the acid resulting from cleavage of the side chain, is under test; I shall not be greatly surprised if the results are negative.

A second vague thought was that the carcinogen might be some unusual or unknown product of oxidation of cholesterol. Additional work on a theme that already has been investigated exhaustively requires not only an incentive but some new method of attack. In their classical paper, Mauthner and Suida (1896) apologized for adding to the already 50-year-old literature on the oxidation of cholesterol and explained that they had conceived the idea of trying a new reagent, chromic

3.6-dione (XVII), easily obtainable in yield of about 35 per cent.

A further product that puzzled me for some little time is prepared most readily by pouring a hot acetic acid solution of two equivalents of sodium dichromate onto a batch of commercial cholesterol. A green solution results in less than a minute, and on concentration of a neutral ethereal extract of the reaction mixture the product separates in almost pure form; one crystallization gives pure material, m.p. 188°, in yield of about 10 per cent by weight. The substance is a 1:1 complex of epicholesterol (XVIII) and 6β-hydroxy-Δ^4-cholestene-3-one (XVI); since it is readily split by chromatography, the reaction constitutes a simple method of preparing epicholesterol. Epicholesterol forms similar, well-crystalline, sharply melting complexes with the 6α-hydroxy epimer, with Δ^4-cholesten-3-one, and with cholestanone, but not with coprostanone. 6β-Hydroxy-Δ^4-cholesten-3-one complexes with epicholestanol, but not with epicoprostanol, cholesterol, or cholestanol

The surprising epimerization of cholesterol in the course of the partial oxidation was observed also in a low-temperature reaction that afforded (after chromatography) an initially mysterious product that proved to be the complex of epicholesterol and Δ^4-cholesten-3-one. The most likely interpretation is that the reaction proceeds through a 3.5-cyclic intermediate comparable to that postulated by Plattner and Lang (1944) (Fig. 4). A similar interpretation is advanced in interpretation of the formation of Windaus's heretofore unidentified "Stoff A" from oxidation of epicholesteryl acetate (Windaus and Naggatz, 1939) which Mary Fieser and E. J. Tarlton have recently shown to be 5α-acetoxycholestane-3.6-dione. The specific mechanism formulated for the chromate epimerization was suggested to me by Dr. D. H. R. Barton.

In carrying out a great many oxidations and chromatographic analyses of the reaction mixtures, I encountered minute amounts of three products that did not seem to be derived from cholesterol (one was later identified as an epichol-

unsaturated diketone, reduced it with zinc and acetic acid to Δ^4-cholesten-3-one, isomerized it to cholestane-3:6-dione, epimerized the acetate to 6α-acetoxy-Δ^4-cholesten-3-one by the method of Herzig and Ehrenstein (1951), and prepared various derivatives. The initial product in the dichromate

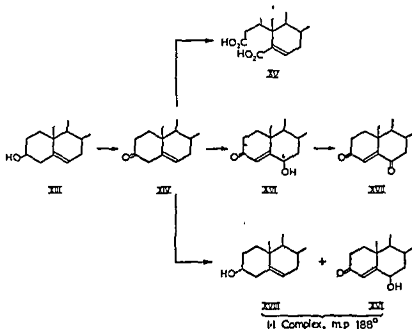
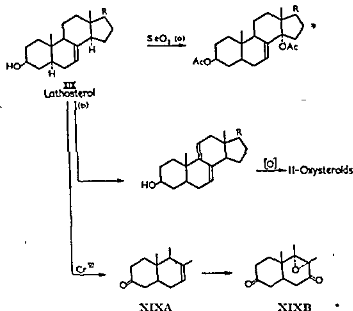


FIG. 3.

oxidation of cholesterol is Δ^5-cholesten-3-one (XIV), which is oxidized very readily to the Diels acid (XV) and to 6β-hydroxy-Δ^4-cholesten-3-one (XVI). This alcoholic product can be isolated readily when a limited amount of dichromate is employed, since it is distinctly less easily attacked than its precursor. It is formed also on dichromate oxidation of cholestenone enol acetate and of Δ^4-cholestene-3β:6β-diol, and on selenium dioxide oxidation of Δ^5-cholesten-3-one. The neutral end product of chromate oxidation is Δ^4-cholestene-

chromatography of the acetates (Fieser, 1951). One was the acetate of cholestanol, the other the acetate of an isomer of cholesterol promptly identified as Δ^7-cholestenol (Windaus and Naggatz, 1939). For convenience in referring to material of biological origin, I suggested the trivial name lathosterol



(a) On XIX as acetate
(b) With $Hg(OAc)_2$, Br_2, *N*-bromosuccinimide or ultra-violet light



$R = C_8H_{17}$

XIXA	Lathostenone	m.p., 145°;	$[\alpha]_D$, +24 7°
XIXB	Diketo-oxide	181°;	−13°

FIG. 5.

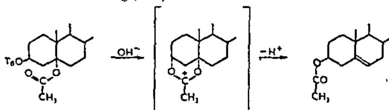
(Gr. *latho*; undetected), and the suggestion has been accepted informally by several workers in the field.

Lathosterol is much more reactive than cholesterol and is characterized particularly by its high sensitivity to oxidation (Fig. 5). It reacts with selenium dioxide in benzene-acetic acid even at 0° and the starting material is completely

*Later work has shown that this is 3β:7α-diacetoxycholest-8(14)-ene.

esterol-ketone complex) and so was prompted to investigate the homogeneity of the commercial cholesterol, which was material of good melting point derived from spinal cord and brain of cattle (Wilson Co.). Since cholestanol is a generally accepted if rather dubiously characterized companion sub-

Plattner and Lang (1944)



Mary Fieser and Tarlton

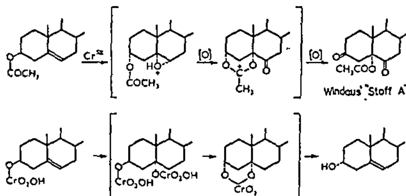
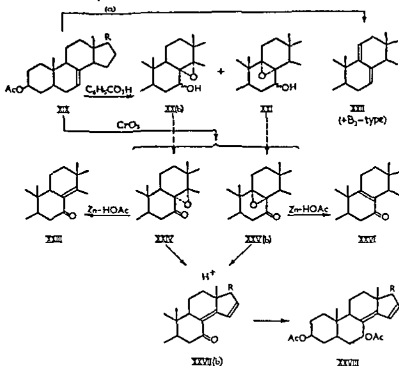


FIG. 4.

stance (Schonheimer, v. Behring, Hummel and Schindel, 1930; Gardner and Gainsborough, 1934), I experimented with mixtures of cholesterol and cholestanol, and found that quick crystallization from acetic acid (as complex) effects considerable concentration of the stanol in the mother liquor. Repeated crystallization of a large batch of commercial cholesterol eventually afforded mother-liquor fractions sufficiently rich in companions for isolation of two pure substances by

Properties of the 7-acetoxy derivative resulting from reaction of lathosteryl acetate with selenium dioxide in benzene-acetic acid are shown in Fig. 7.

L. F. Fieser, Herz and Nakanishi



(a) With $Hg(OAc)_2$, SeO_2, peracids, N-bromosuccinimide or Br_2
(b) cf. Wintersteiner and Moore (1943)

$R = C_8H_{17}$

XXVIII has λ_{max}^{EtOH} 247 5 mμ.

FIG. 6

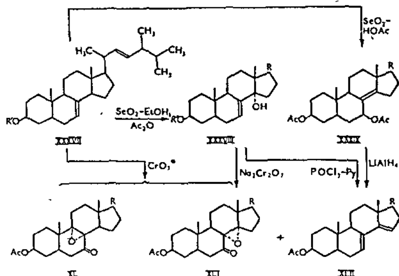
Contrary to the conclusion of Callow and Rosenheim (1933), my collaborator Guy Ourisson has found that the product of oxidation of 5-dihydroergosterol (Fig. 8, XXXVII) in ethanol is not an oxide but the 14α-hydroxy compound (XXXVIII),

consumed in one hour; a similar solution containing cholesterol is completely colourless after twenty-four hours at 0° and shows evidence of reaction only at 50–60°. By a simple micro test conducted in a melting point capillary with 1 mg. of material, one can easily differentiate between gallstone cholesterol (2–3 per cent lathosterol) and the same material purified through the dibromide (0 per cent). Baumann (Moore and Baumann, 1952; Idler and Baumann, 1952), who recently has found that lathosterol is an abundant component of skin sterols, has observed that this isomer enters into the Liebermann-Burchard reaction much more rapidly than cholesterol and can be assayed colorimetrically in the presence of cholesterol; the reaction is also applicable as a qualitative micro test. Lathosterol is dehydrogenated by a variety of mild oxidizing agents to $\Delta^{7,9(11)}$-cholestadien-3β-ol, known from the recent work on the cortisone problem to be convertible on further oxidation into 11-oxygenated sterols of various types. In view of the special physiological functions associated with the 11-oxygenated cortical hormones, it is an attractive speculation to suppose that some such product may be a carcinogen and that, under a coincidence of circumstances, it might arise in the body.

Chromate oxidation of lathosterol gave the 3-ketone (XIXA) and a diketo oxide (XIXB) both different from the unidentified products derived from commercial cholesterol. Chromate oxidation of the acetate (Fig. 6) gave the 7-keto-8:9-oxide (XXV) of Wintersteiner and Moore (1943) and the isomeric 7-keto-8:14-oxide (XXIV); these were found reducible with zinc and acetic acid to the corresponding unsaturated ketones (XXVI and XXIII) which are stable to chromate oxidation. Perbenzoic acid reacted with lathosterol to give both the known 7-hydroxy-8.14-oxide (XX) and the isomeric 8 9-oxide (XXI). The structure of the dienone resulting on acid hydrolysis of both ketoxides has been the subject of uncertainty. Reduction of the carbonyl group with lithium aluminium hydride gave a product having an absorption maximum indicative of the structure formulated.

suggests a mechanism for the production of the latter substance on oxidation of the Δ^7-stenol.†

The results summarized in Table I show that low-temperature oxidation by selenium dioxide in acetic acid-benzene is



Rotations, $[M]_D$, in $CHCl_3$. Absorption maxima λ_{max} ($m\mu$) in EtOH
R (side-chain) = C_9H_{17}

	$[M]_D$	λ_{max}	ϵ_{max}
XXXVII a, R′ = H	−80		
XXXVII b, R′ = Ac	−88		
XXXVIII a, R′ = H	−184		
XXXVIII b, R′ = Ac	−214		
XXXIX	−192		
XL	−216		
XLI	−466		
XLII	−952	242	10,400

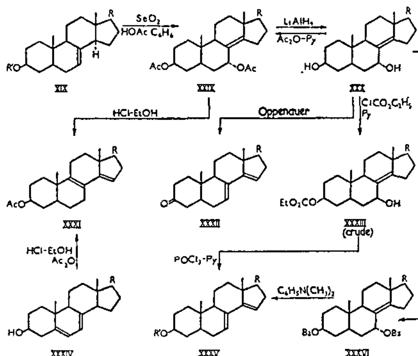
FIG. 8.

*Stavely & Bollenback

specific to steroids of the 5a- or the Δ^5-series having a double bond adjacent to the 14a-hydrogen atom; apparently attack of an activated tertiary hydrogen atom occurs only if the

†Later work has shown the structure XXXVIII to be incorrect; the substance actually is 7a-ethoxy-$\Delta^{8(14),22}$-ergostadiene-3β-ol.

as evidenced by dehydration under strictly non-acidic conditions to the B_3-type 7:14 diene (XLII.) Chromic acid oxidation of the allylic alcohol to the 7-keto-8:14-oxide (XLI)



Rotations, $[M]_D$, in $CHCl_3$, Absorption maxima λ_{max} ($m\mu$) in EtOH
R (side-chain) = C_8H_{17}

	$[M]_D$	λ_{max}	ϵ_{max}
XIX a, R' = H	+15°		
XIX b, R' = Ac	+8		
XIX c, R' = Bz	+34		
XXIX	−20		
XXX	−86		
XXXI	−90	250	18,100
XXXII	−563	242	9,700
XXXV a, R' = CO_2Et	−737	242	9,800
XXXV b, R' = Bz	−733		

FIG. 7.

residue with nitric acid to destroy the sterol and convert the selenium into selenious acid, destroying nitrous oxides with sulphamic acid, adding excess potassium iodide, and determining the iodine spectrophotometrically. The result gives the total content of lathosterol and 7-dehydrocholesterol, but in sterols derived from sources other than skin the amount of diene present is negligible. The results of an exploratory study of various tissues are suggestive of interesting differences in Δ^7-stenol content (Table II).

Table II

LATHOSTEROL CONTENT OF "CHOLESTEROL"

Samples prepared by Bidyut Bhattacharyya	Δ^7-*Stenol per cent*
Commercial cholesterol (Wilson, spinal cord and brain)	0 62
Gallstone cholesterol	2 19 to 3 11
Egg yolk cholesterol	0 39
Beef adrenal cholesterol	0 65
[illegible]	0 35
[illegible]	2 00
[illegible]	2 97
[illegible]	1 43
[illegible]	0 60
[illegible]	2 93
[illegible]	2 49
[illegible]	0 29
[illegible]	0 10, 0 10
S_f 4-8 Fractions from same patient	0 29
All other fractions, combined	0 24

*S_f=sedimentation fractions in the ultracentrifuge

It is interesting that cholestanol and lathosterol are the 5.6-dihydro derivatives of cholesterol and provitamin D_3, respectively (Table III). There are some suggestions that another as-yet-unisolated companion that I very tentatively call thaptosterol (Gr. *thapto*, buried) is a *seco*sterol related to vitamin D_3 rather than to a *tetracyclic sterol proper*. After fruitless attempts to isolate a merely suspected further companion of unknown properties, I perfected a method of oxidative analysis by which an oxidation product of the companion can be isolated with comparative ease. A 20-g.

latter is rear-oriented, and it is inhibited by the shielding effect of the *A/B-cis* configuration. I am indebted to Drs. Rosenkranz, Djerassi, and Barton for tests conducted in Mexico City and in London.

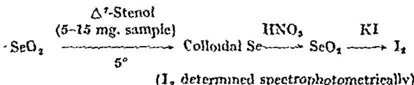
Table I

SELENIUM DIOXIDE TEST

Sample (1 mg.) in benzene (0·5 ml.); 0·5 ml. 0·1 M H_2SeO_3 in HOAc—H_2O added at 25°. Positive reaction, yellow in 2-3 min., red deposit of Se in 10-15 min. Negative reaction, colourless after 1 hr.

Configuration at $C_{(5)}$	*Position of Double bond(s)*	*Number of compounds tested*
Positive reaction		
5α	7	7
5α	8(9)	2
5α	7 9(11)	3
—	5 7	4
5α	6 8(9)	2
Negative Reaction		
5β	7	3
5β	8(9)	1
5α or 5β	9(11)	3
5α or 5β	8(14)	5
5α or 5β	14	3
5β	7·9(11)	2
5α or 5β	8(9).14	2
5α	6 8(14)	2

A micro method of determining Δ^7-stenols worked out by Koji Nakanishi



consists in treating a small sample with selenium dioxide in benzene-acetic acid at 5°, washing out the excess selenious acid with bicarbonate, evaporating the benzene extract containing colloidal selenium in a Carius tube, heating the

containing small amounts of added lathosterol and cholestanol and obtained high recoveries of oily latho fraction (100 per cent) and of pure cholestanone (80 per cent). Table IV shows the yields per 20 g. of total sterol of the new product, here designated Ketone 104.

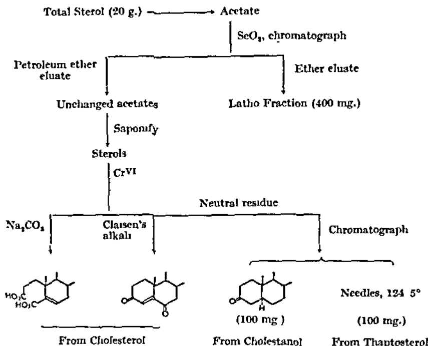


FIG. 9. Analysis of Tissue "Cholesterol".

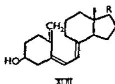
The precursor substance follows cholesterol through the process of purification by way of the dibromide (which completely eliminates lathosterol and cholestanol), and the yield from dibromide-purified sterol is over twice that from the original total sterol. Dr. Bhattacharyya and I processed cholesterol from a variety of sources in the hope of finding a source particularly rich in the unknown precursor, but thus far all

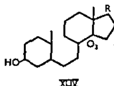
portion of sterol is treated in the form of the acetate with selenium dioxide at 25° and then chromatographed (Fig. 9). The lathosteryl acetate present is quantitatively converted into a mixture of strongly adsorbed products, whereas cholesteryl and cholestanyl acetate are unattacked and are quantitatively recovered by elution with petroleum ether. The latter fraction is saponified and oxidized overnight at 7–13° in

Table III

COMPONENTS OF "CHOLESTEROL"

Cholesterol	Cholest-5-en-3β-ol
Provitamin D_3	Cholesta-5 7-dien-3β-ol (0·18 per cent in egg-yolk sterol)
Vitamin D_3	9 10-*seco*-Cholesta-5.7 10(19)-trien-3β-ol (XLIII)
Cholestanol	Cholestan-3β-ol (0 2-0 5 per cent)
Lathosterol	Cholest-7-en-3β-ol (0·5-3 per cent)
Thaptosterol	? 9 10-*seco*-Cholestan-3β-ol with two additional O atoms (0 5 per cent, XLIV)





benzene-acetic acid with 10 oxygen equivalents of dichromate. About half of the cholesterol present is oxidized to acids (Diels acid, 7-keto-Diels acid) extractable with soda, and the rest affords Δ^4-cholestene-3:6-dione, which can be quantitatively removed by extraction from hexane with Claisen's alkali (aqueous methanolic KOH). The small neutral residue (0·4–0·6 g.) is chromatographed on alumina, when petroleum ether (90)-benzene (10) elutes first cholestanone and then an

samples have afforded Ketone 104 in yields ranging from 75–123 mg./20 g. Incidentally, Dr. Bhattacharyya has isolated lathosterol, cholestanol, and cholestane-3β·5α:6β-triol as such from gallstone cholesterol, and the triol also from egg yolk and brain cholesterol.

The data of Fig. 10 show that according to four closely agreeing analyses (of three separate samples) Ketone 104 has the formula $C_{27}H_{44}O_3$; analyses of three crystalline derivatives confirm the formula. A triketo or keto-dioxide derivative of cholestane would have the formula $C_{27}H_{42}O_3$, and hence the substance, if indeed it is a steroid, would appear to be a *seco*-steroid derivative. Dr. Bhattacharyya reduced the ketone to an alcohol that has failed to crystallize but that yields Ketone 104 on reoxidation and affords a crystalline acetate from which the liquid alcohol is recovered on saponification. Like vitamins D_2 and D_3 and their hydro derivatives, the liquid alcohol gives no precipitate with digitonin. Formation of the monoethylene mercaptal of Ketone 104 is attended with disappearance of the carbonyl band at 5 81 μ, and there is no suggestion in the infra-red spectrum of the presence of any other active-oxygen function. The bands in the region 9–11 μ suggest that the two inert oxygen atoms are present as oxide linkages. The oxidic rings are very stable and have thus far resisted all attempts to effect cleavage. A sapogenin-type structure seems excluded, since methods of fission, oxidation, and reduction applicable to sapogenins are not applicable here.

Just before my departure for this conference (via a symposium in Jerusalem), Drs. Bhattacharyya and Wei-Yuan Huang and I initiated experiments with ^{14}C-labelled cholesterol in order to explore the relations between the various companions and in the hope of finding a means of following thaptosterol in the course of fractionations. Table V presents the results of an exploratory experiment in which the method of oxidative analysis (Fig. 9) was applied to the mixture shown, containing ^{14}C-cholesterol derived from injection of $^{14}CH_3CO_2Na$ into a rat that was killed four hours later. The activities of pure

Table IV

CHOLESTEROL FROM VARIOUS SOURCES

Source	Companions or Oxidation Products (mg./20 g.)				
	Lathosterol	Ketone 104	Cholestanol	Cholestanetriol	Cholestanedionol
Spinal cord and brain of cattle (Wilson) Old lot, cryst. from HOAc Same, through dibromide New lot, through dibromide Very old lot, through dibromide	Isolated	40-47 115 83 123	Isolated		? ? 410 440
Gallstones	Isolated	100*	Isolated	Isolated	?
Human brain		120*		Isolated	
Egg yolk		108*		Isolated	?
Wool fat		103*			?
Mollusk Sterol (less brassicasterol)		136*			?
Shark liver oil, South American		100*			339
Skipjack liver oil		86*			436
Whale liver oil		75*			?

* After purification through dibromide

samples have afforded Ketone 104 in yields ranging from 75–123 mg./20 g. Incidentally, Dr. Bhattacharyya has isolated lathosterol, cholestanol, and cholestane-3β:5α:6β-triol as such from gallstone cholesterol, and the triol also from egg yolk and brain cholesterol.

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Spinal cord and brain of cattle (Wilson) Old lot, cryst. from HOAc Same, through dibromide New lot, through dibromide Very old lot, through dibromide	Isolated	40 47 115 85 123	Isolated	.	? ? 410 440
Gallstones	Isolated	100*	Isolated	Isolated	?
Human brain		120*	'	Isolated	
Egg yolk		108*		Isolated	?
Wool fat		103*			?
Mollusk Sterol (less brassicasterol)		136*			?
Shark liver oil, South American		106*			339
Skipjack liver oil		86*			430
Whale liver oil		75*			?

* After purification through dibromide

crystalline Δ^4-cholestene-3.6-dione, Diels acid monomethyl ester, and cholestane-3.6-dion-5α-ol were all about the same as that of the total sterol, and the activity of the unseparated

Table V

DISTRIBUTION OF RADIOACTIVITY ON FRACTIONATION

Mixture of 10 g of Dibromide-purified Cholesterol, 227 mg. of Biosynthetic ^{14}C-Cholesterol, 50 mg of Cholestanol, and 100 mg of Lathosterol

	Counts/min/mg
Total mixture	40
[illegible]	54
[illegible]	45
[illegible]	26
[illegible]	34
Ketone fraction: Cholestanone and $C_{27}H_{44}O_2$	39
Lathosterol fraction (oil)	180

ketone fraction was also in the same range. The lathosterol fraction, however, proved to be four times as active as the cholesterol derivatives.

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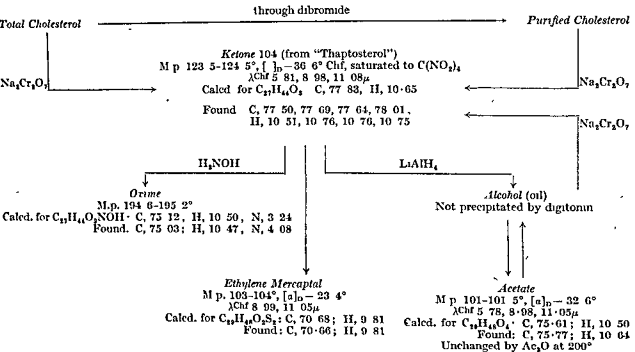
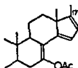
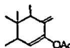

through dibromide
Total Cholesterol
Purified Cholesterol
$Na_2Cr_2O_7$
$Na_2Cr_2O_7$
$Na_2Cr_2O_7$
Ketone 104 (from "Thaptosterol")
M p 123 5-124 5°, [ ]$_D$ −36 6° Chf, saturated to $C(NO_2)_4$
λChf 5 81, 8 98, 11 08μ
Calcd for $C_{27}H_{44}O_3$ C, 77 83, H, 10·65
Found C, 77 50, 77 69, 77 64, 78 01, H, 10 51, 10 76, 10 76, 10 75
H_2NOH
LiAlH$_4$
Oxime
M.p. 194 6-195 2°
Calcd. for $C_{27}H_{44}O_2NOH$ · C, 75 12, H, 10 50, N, 3 24
Found. C, 75 03; H, 10 47, N, 4 08
Alcohol (oil)
Not precipitated by digitonin
Ethylene Mercaptal
M p. 103-104°, [α]$_D$ − 23 4°
λChf 8 99, 11 05μ
Calcd. for $C_{29}H_{48}O_2S_2$: C, 70 68; H, 9 81
Found: C, 70·66; H, 9 81
Acetate
M p 101-101 5°, [α]$_D$ − 32 6°
λChf 5 78, 8·98, 11·05μ
Calcd. for $C_{29}H_{48}O_4$ · C, 75·61; H, 10 50
Found: C, 75·77; H, 10 64
Unchanged by Ac_2O at 200°

Fig. 10.

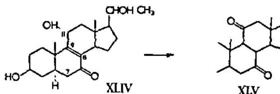
calculated for XXVII. Two other structures are possible but calculations cannot be made because they are cross-conjugated. But by reducing the 7-keto group we eliminated some of the difficulty, and could definitely rule out one of the formulæ. We had hoped that the



XLII



XLIII



XLIV XLV

DJERASSI: Yes, I was thinking of our dehydration product of 5α-pregn-8(9)-ene-3β 11α 20β-triol-7-one (XLIV) (*J. Amer. chem Soc.*, 1952, 74, 2918) While the important isomerization of the unsaturated ketol XLIV to the saturated 7 11-dione XLV proceeds quite readily

system (XLVI) should absorb around 315 mμ. Hence our compound could have one of the two other obvious structures, a cross-conjugated 8(14) 9(11)-dien-7-one (XLVII) or your structure (XXVII)

FIESER: Structure XLVI is a possible formulation for the compound in question, although there is evidence that the substance is converted on hydrogenation into cholest-8(14)-en-3β-ol-7-one acetate I suppose it is possible that XLVI suffers saturation at 11(12) and that the double bond migrates under the influence of the catalyst from 8(9) to 8(14), but this seems unlikely.

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DISCUSSION

MARRIAN It will be very interesting when some of these accompanying substances are tested biologically. Are you starting on that now, Dr. Fieser?

FIESER Not being a biologist or biochemist I can do very little except to urge some of my friends to take up work of this kind, and I think I'm having some success in that

MARRIAN: An exhaustive examination of cholesterol oxidized by

to have prepared highly carcinogenic materials. Bergmann repeated the work and had the materials tested, the results were negative

SONDHEIMER Dr Wettstein (*Helv. chim. Acta*, 1940, 23, 388) found some time ago that on oxidizing cholesterol or other Δ^{5}-3-alcohols by the Oppenauer method under *special conditions he obtained the* $\Delta^{4,6}$-dienone by a reaction which probably proceeds through the Δ^{5}-3-*ketone. Recently we found at Syntex that the same result is obtained* with manganese dioxide. I wondered whether you obtained $\Delta^{4,6}$-dienones in the oxidation of cholesterol by your methods. It would be easy to detect very small amounts by the ultra-violet spectrum.

FIESER I don't think so, because if I use enough dichromate to completely exhaust the cholesterol and completely oxidize the 6β- ... then I get in the neutral fraction very pure ... ntaminated with any- ... ation and sh on, and I ... ne. I don't think it's formed.

DJERASSI. Do you happen to remember the ultra-violet absorption maximum of your 7-keto-$\Delta^{8(14),15(16)}$-diene (XXVII)?

FIESER: We checked Stavely and Bollenback's compound and the maximum is around 298 mμ, which agrees with the value of 295 mμ

for unsaturation, Liebermann-Burchard tests, and so on. It's a nice technique. When you're through the test you throw away your test-tubes instead of washing them.

PINCUS: Dr. Fieser is aware of some work with cholesterol derived from acetate that is being done by Dr. Schwenk in our laboratories. I just want to mention two aspects of it. First of all, he more or less confirmed the work reported in Table V. Instead of oxidation products Dr. Schwenk has been studying the products of saponification of livers perfused with radioacetate, and he obtains certain chromato-

[illegible]

which had a high count. I was wondering when your rat was killed.

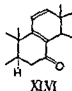
FIESER: Ours was killed 4 hours after injection, and this was only one rat. We want to do a lot of experiments, allowing varying periods of incubation Only then can we get data which can be interpreted I just mentioned this as preliminary work.

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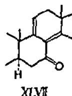
FIESER Of course we started this work partly in the hope that this Ketone 104 would show some striking difference in radioactivity, and that we might therefore use that method as a means of following its

[illegible]

FIESER: I don't know. It seems to me more likely that it goes through some kind of hemiketal affair It goes quite smoothly and you get pretty good yields It's very specific and gives only one of the two possible isomers.



XLVI



XLVII

DJERASSI This is not very important because it is only a by-product. Our structure is quite conceivably wrong *

SHOPPEE: I should like to mention briefly some work which I did about 5 years ago at the Royal Cancer Hospital in London. I was presented with some 30 g. of an unsaponifiable fraction derived from Bantu livers. Bantu are particularly susceptible to primary cancer of the liver. This material had been subjected to very brutal treatment, boiling with potassium hydroxide, without exclusion of atmospheric oxygen I proceeded to try and find out what was in it. I isolated a hydrocarbon, the C_{31}-n-paraffin, a triol (the cholestane-3β.5α·6β-triol, which Professor Fieser mentioned a moment ago) and, if my memory serves me right, the 7-hydroxy- and 7-keto-cholesterols.

In trying to check the purity of the various cholesteryl acetate fractions chromatographically I used a colour reaction with $SbCl_3$ in chloroform under carefully controlled conditions. The purest fractions of cholesteryl acetate failed to give any colour; but quite a large number of fractions did develop a pink colour with this reagent. I am now wondering in retrospect if in fact some of these fractions did contain small quantities, which I was unable to separate, of Professor Fieser's lathosterol I asked him privately if he had any information about this particular colour reaction, and he has a suspicion that his lathosterol does in fact give a pink colour with this reagent.

FIESER I haven't gone very far with colour tests on lathosterol because we found the selenium dioxide reaction is so good and so

column of liquid.

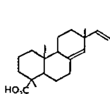
You can use the same method for doing tests with tetranitromethane

*Structure XXVII has since been shown to be the correct one (Lemin, Rosenkranz and Djerassi, 1953, *J. Amer. chem. Soc*, 75, In Press).

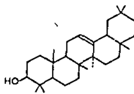
THE CHEMISTRY OF LANOSTEROL

D. H. R. BARTON

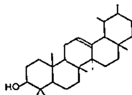
The discovery of the outstanding medicinal value of cortisone initiated a great deal of research into methods for its partial synthesis from readily available starting materials. Much of the elegant steroid chemistry arising from this work will be summarized in subsequent contributions to this Conference. Naturally consideration has also been given to the possibility of a partial synthesis from non-steroid materials of which, for example, dextropimaric acid (I), amongst diterpenoid compounds, might be a conceivable precursor.



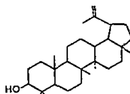
I



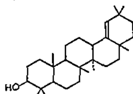
II



III



IV



V

COOK. It would be interesting to make an enol methyl ether and then reduce it. That might give some information about the mechanism

The question I wanted to put to Professor Shoppee was in regard to the possible carcinogenicity of by-products in cholesterol. I think that Hieger made some biological tests with very highly purified cholesterol and still obtained tumours. Professor Shoppee, I think, took part in that work and possibly could tell us something about it. I've always been uneasy about this work on the carcinogenic action of cholesterol and such widespread natural products because, to the best of my knowledge, all the tumours have been obtained by using cholesterol dissolved in vegetable oils or animal oils of uncertain composition. I know that controls were done in some of these experiments, but even so in some of the controls tumours were obtained. I'm not very sure that we're not chasing a hare in looking for carcinogenic properties in cholesterol I would like to know from Prof Shoppee whether his very highly purified cholesterol was found to give tumours

SHOPPEE This question relates to events of 5 years ago but I will try to say what I can. I made for Dr. Hieger some highly purified cholesterol by putting it first through dibromide, and then making the acetate, and doing a chromatographic separation into a large number of fractions, and rejecting all those which gave the colour test which I mentioned previously (p 24) The product, which I regarded hopefully as a really pure cholesteryl acetate, was then hydrolysed carefully with alkali, and this was shot into animals I think Dr. Hieger got one tumour out of a very old animal right at the end of the series of experiments. I don't think it's safe to draw any conclusions, the appearance of that tumour upset the whole box of tricks On the other hand, I think he regarded the fact that he got no tumours out of all the controls as an indication that his solvent—I think it was lard—was above suspicion

COOK. I see But he did get a much higher incidence of tumours with ordinary cholesterol, didn't he?

SHOPPEE He used a whole series of unpurified cholesterols and had sarcomas in quite a number of cases.

FIESER But this was one tumour out of 25 that he got with your pure cholesterol

SHOPPEE: Yes, right at the end of the series.

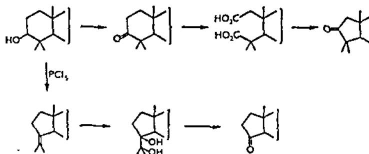
FIESER With crude cholesterol, Hieger's average incidence of tumours is 4 per cent.

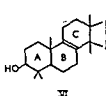
PINCUS One out of 25 is 4 per cent!

SHOPPEE: Dr. Hieger wanted me to make a really large quantity of this so-called highly purified material so that he could repeat this with several hundred mice, but I just never found the time or the energy to undertake this.

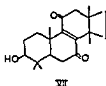
FIESER I've been working on cholesterol for years and I don't know how to make pure cholesterol or how to tell whether it's completely homogeneous. If you purify it through the dibromide it still contains the substance that I call thaptosterol But at least I think we can do better than to use garden variety cholesterol or crude lipids from liver dissolved in lard and purified by letting some of it drip through a funnel at 37°!

Dorée, McGhie and Kurzer, 1948, 1949*b*; Voser, Montavon, Günthard, Jeger and Ruzicka, 1950). Further oxidation with selenium dioxide affords successively dehydrodiketolanostenyl acetate and dehydrotriketolanostenyl acetate (VIII),

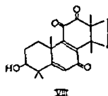




VI
Lanostenol

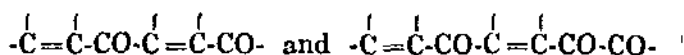


VII
Diketolanostenol

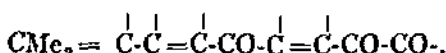


VIII
Dehydrotriketolanostenol

compounds which contain, respectively, the chromophoric systems—



(Dorée, McGhie and Kurzer, 1948, 1949; Voser *et al.*, 1950). The relationship between the secondary hydroxyl group and the latter chromophore has been demonstrated by the fact that treatment with phosphorus pentachloride affords an anhydro- compound (IX) which contains the system.—



Further oxidation with alkaline hydrogen peroxide gives a compound (X) having the chromophoric system:—

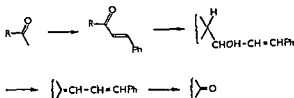
The brilliant researches of L. Ruzicka, O. Jeger, and their collaborators in Zürich on the chemistry of the triterpenoids, compounds containing 30 carbon atoms, which occur abundantly in nature, have led to the elucidation of the constitutions of most of the pentacyclic members. For example we have β-amyrin (II), α-amyrin (III), lupeol (IV) and germanicol (V). Obviously these substances are not readily adapted to conversion into cortisone or cortisone-like compounds. Another large group of triterpenoids contains four carbocylic rings. Up to two years ago these compounds had received little attention and, since no constitutional formulæ had been proposed, it was not possible to evaluate their potentialities as cortisone precursors. One of the most abundant of the tetracyclic triterpenoids is lanosterol (lanostadienol), obtainable in large amounts, along with cholesterol, from the non-saponifiable matter of wool fat.

For our knowledge of the constitution of lanosterol we are indebted, in the main part, to the very elegant investigations of L. Ruzicka, O. Jeger, and their colleagues extending over a decade. Important contributions have also been made by J. F. McGhie and his associates at Chelsea Polytechnic, London.

Lanosterol, $C_{30}H_{50}O$, has one secondary hydroxyl group and two double bonds. The secondary hydroxyl group is flanked by $-CH_2-$ and by $-CMe_2-$. This is shown by the reactions outlined below (Ruzicka, Rey and Muhr, 1944; Ruzicka, Montavon and Jeger, 1948, Dorée, McGhie and Kurzer, 1949*a*). These reactions also indicate that the hydroxyl is contained in a six-membered ring.

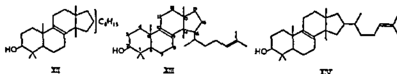
One of the ethylenic linkages is readily hydrogenated and has been shown to be present as the grouping $-CH=CMe_2$. The other ethylenic linkage is inert and, from infra-red evidence (Roth and Jeger, 1949) is tetrasubstituted. Oxidation of dihydrolanosteryl (lanostenyl) acetate (VI) with chromic acid gives a yellow diketo-compound (VII) containing the grouping -CO-C=C-CO- in the fully *transoid* arrangement (Ruzicka, Rey and Muhr, 1944; Dorée and McGhie, 1944;

The size of the fourth ring in lanosterol was first proved by Voser, Günthard, Jeger and Ruzicka (1952) who devised an extension of their side chain degradation in order to attain the *D*-ring ketone. The relevant reactions can be summarized as indicated.



The carbonyl frequency of the *D*-ring ketone showed that it must be present in a five-membered ring.

On the basis of all this evidence the formula of lanosterol can be expressed as in (XII). If the carbon skeleton of lanosterol be assumed to conform to the "isoprene rule"—a "rule" which has seen much sterling service in the classical investigations of Ruzicka and his school in the field of terpenoid chemistry—then only two formulæ, (XIII) and (XIV), are possible.



A distinction between these formulæ has been achieved by the reactions indicated below (Barnes, Barton, Cole, Fawcett and Thomas, 1952):—

The triacetoxylananone (XVI) was shown to have the grouping -CO-CH_2-CH_2- rather than -CH_2-CO-CH_2- on the basis of steric hindrance evidence, quantitative bromination studies *and the intensity of the band at 1,410 cm.*$^{-1}$ *characteristic* of the grouping -CH_2- adjacent to a keto-group in a five-membered ring. All these observations were, of course, coupled with the appropriate experiments on model compounds.

$$CMe_2=\overset{|}{C}\text{-}\overset{|}{C}=\overset{|}{C}\text{-}CO\text{-}\overset{|}{C}=\overset{|}{C}\text{-}CO_2H\ \ HO_2C\text{-}$$

(Barton, Fawcett and Thomas, 1951; see also Cavalla, McGhie and Pradhan, 1951). Now dehydrogenation of lanosterol (Schulze, 1936; Ruzicka, Rey and Muhr, 1944) affords 1:2:8-trimethylphenanthrene (XI), a hydrocarbon which is obtained in a similar way from most of the tetracyclic triterpenoids. Placing the chromophores indicated above within a phenanthrene framework leads to the partial expressions below.

IX
Anhydro-Compound

X
Fission product of anhydro-compound

XI

Three methyl groups have been placed in angular positions in order to explain the locking of rings A and B, the absence of α-hydrogen in dehydrotriketolanostenol and the production of (XI) on dehydrogenation. Further justification for the assignments comes in the sequel.

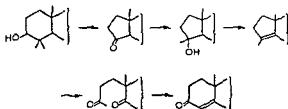
The nature of the secondary octyl side chain in lanosterol was first demonstrated by Voser, Mijovic, Jeger and Ruzicka (1951) by an elegant step-wise degradation of the Meystre-Miescher type. The relevant reactions were as indicated.

$$\rightarrow RCOCH_3 \quad \rightarrow RCO_2H$$

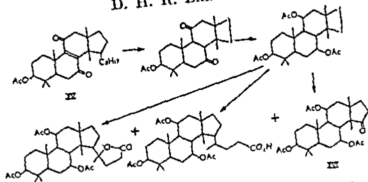
The structure of the side chain is also shown by the isolation of 6-methylheptan-2-one as a by-product of vigorous chromic acid oxidation of lanostenyl acetate (Barton, McGhie *et al.*, 1951).

the introduction of a keto group at C_{15} should cause a large negative rotation if the *C/D* fusion were *cis*. If this fusion were *trans* then the introduction of the ketone grouping should cause a positive rotational increment of a magnitude comparable to that produced by the C_{17} keto-group in steroids (+250). Triacetoxylanane has been prepared from the ketone (XVI) and the rotational contribution of the ketone grouping shown to be +120. Rings *C* and *D* must, therefore, be fused *trans*. It has been shown (McGhie, 1952) that the side chain of lanosterol is fused to the lanane nucleus in the more stable configuration. On the basis of the concept of equatorial and polar bonds (Barton, 1950) this means that the attachment should be *α*. In agreement the contribution of the side chain of triacetoxylanostane to the molecular rotation is +90 which is in excellent agreement, as it should be on the basis of the generalized Molecular Rotation Difference Method, with the 17*β*-oriented side chain of cholestanol (+86).

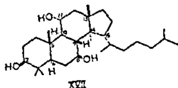
On the basis of the work summarized above both the similarities and the dissimilarities of lanosterol with respect to the normal steroid molecule can be assessed. It is clear that the presence of the C_{14} methyl group will prevent any direct conversion to a steroid. The 9(10) double bond, on the other hand, is ideally suited to the introduction of ketonic oxygen at C_{11} and, in a recent paper, Voser, White, Heusser, Jeger and Ruzicka (1952) have given an elegant demonstration of how ring *A* can be modified to give a six-membered *αβ*-unsaturated ketone thus:—



The way is now clear for the synthesis of analogues of cortisone based on lanosterol



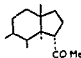
The probable stereochemistry of trihydroxylanostane is indicated in (XVII). The arguments in favour of this



are briefly as follows From application of his generalized Molecular Rotation Difference Method, Klyne (1952) has concluded that rings *A* and *B* are *trans* fused and that the C_5 methyl group has the same configuration in space as the C_{10} methyl groups in steroids and the C_5 methyl group in the pentacyclic triterpenoids. The hydroxyl must be on the same side (β) as the C_5 methyl group, on the basis of arguments adduced elsewhere. The methyl group at C_{13} is placed β in order to explain the high degree of steric hindrance shown by the polar type hydroxyl at C_{11}. The degree of hindrance is the same as that shown by 11β-hydroxy compounds in the steroid series, where the hindrance is likewise due to two polar type methyl groups. The configurations at C_9 and C_{10} in the lanostane type compounds are assigned as indicated on the grounds that these centres must be in the more stable configurations because of the mode of preparation. The configuration at C_{14} is assigned on the basis of generalized Molecular Rotation Arguments. Thus, according to Klyne,

SARETT: We've studied some total-synthetic intermediates with hydroxyl groups that are analogous to the 11β-hydroxyl, and they exhibit considerable steric hindrance. They can be acetylated with

FIESER: Dr. Barton, has no natural product yet violated the isoprene rule?



XVIII

BARTON. The great majority of terpenoids obey the isoprene rule. There are exceptions known—but not, as yet, in triterpene chemistry. The circumstantial evidence in favour of attaching the side chain to C-15 is, I think, quite good. The most compelling argument, although still circumstantial, is, that the methyl ketones (XVIII) that one can prepare by the side chain degradation contain a very sterically

BARTON Experiments are, of course, in hand to obtain a final decision about the attachment of the side chain at C-15 or C-17. I would not consider this steric hindrance argument as something to be

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[illegible] RUZICKA,

[illegible] KA, L.

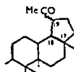
DISCUSSION

SARETT: Dr Barton, what was your criterion of steric hindrance for *the 11-hydroxy group? You said it was hindered in one configuration* and not in the other

BARTON: Yes. If you take the 11-keto group in the lanosterol series and reduce it with $LiAlH_4$ you get a hydroxyl, a polar type hydroxyl according to our views, which is as hindered as the 11β-hydroxyl in steroids. If however you reduce the keto group with sodium in alcohol —that is a procedure which on our views will give the equatorial hydroxyl—then you get a hydroxyl which is readily acetylated.

SARETT: How hindered precisely is the 11β-hydroxyl that you get [illegible]

two opin-
isoprene
with the

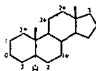


XIX

nificantly hindered

Spring My question was really this, what sort of hindering effect

the keto-group and the number of polar-type methyl groups attached to the β-carbons with respect to the keto-group, and in addition allow a certain increment (+) for when the γ-carbon is bent round upon the keto-group, then the numbers indicated in (XX) are obtained.



XX

If the number is greater than 3 then one has a strongly hindered ketone group.

Reichstein Does it work also for the 20-keto group in steroids of the abnormal series with a 17α-side chain?

Barton Do you mean with the 17β-hydroxyl as well?

Reichstein Even without. The hydroxyl doesn't play a very great rôle. The keto group is much more hindered if the side chain is in the α-position.

Barton: The side chain was placed at C-17 in the formulæ, but with the reservation that it might be attached at 15, 16, or 17. Perhaps Dr Heusser would like to comment?

Djerassi: Do you prefer C-17, Dr. Heusser?

Heusser: Yes.

Djerassi: This would constitute a violation of the isoprene rule

Heusser: Yes. We believe that the methyl group on C-14 has an influence on the ketone group at C-17 in the *trans C/D* series. We don't believe that the point of attachment of the long side chain is really C-15.

Barton: What about the methyl ketone, Dr. Heusser? Will your atomic models tell you that it can be hindered in the same way?

Heusser: It is sterically hindered, but not so strongly that it is a reason for making a definite assignment of the position of the side chain

Barton: That is very interesting I have been careful in our publications (see above) to present the constitutional argument only within the framework of the isoprene rule and with the proper reservations. More experimental work is needed to find out if the side chain is really attached at C-15 or C-17

Cook: You get into rather deep water when you apply the isoprene

It should be possible to say which of the two positions, C-15 or C-17, is consistent with the dimensions of the unit cell.

Barton: As I understand it, Professor Cook, the iodoacetate of

Cholesterol and lanosterol occur together in wool fat, and they're very difficult to separate from one another.

Barton: There is squalene, which occurs in shark oil, and there is ambrein which is produced by the whale. They are both animal products.

Pincus: Dr. Bloch has recently reported that the administration of ^{14}C-squalene gives rise to ^{14}C-cholesterol.

Barton: Yes, I saw that. It's a very remarkable and important

THE ERGOSTEROL ROUTE TO ADRENAL CORTICAL HORMONES

H. B. HENBEST and E. R. H. JONES

SEEKING for routes from ergosterol to compounds related to cortisone, attention was directed to dehydroergosterol peroxide (I) (or epidioxide) as a starting material. The 8-oxygen substituent and the 9:11-bond offered promise of facilitating the introduction of an 11-oxygen atom; the 5-oxygen substituent suggested a convenient way of overcoming the major problem of forming the 3-keto-Δ^4-system; and the 22:23-double bond, provided it could be retained until an appropriate stage, could be used for side-chain degradation and elaboration.

The yield in the photo-oxidation of dehydroergosterol has been considerably increased, from 30 up to nearly 80 per cent, by working under alkaline conditions, thus protecting the acid-labile epidioxide system; and the α-configuration of the epidioxide bridge, as suggested by Fieser (1950) for the ergosterol derivative, has been confirmed.

The original scheme envisaged the reductive fission of the epidioxide bridge to a $\Delta^{9\,11}$-5 8-diol, which might then undergo either hydrogenolysis at $C_{(8)}$, leaving a 9.11-double bond to be oxygenated, or anionotropic rearrangement to an 11-hydroxy-$\Delta^{8\,9}$-compound. No difficulty was anticipated in retaining the 5-hydroxyl group, but the preservation of the side-chain double bond prior to fission set a considerable problem. This was eventually solved following a detailed study of the partial hydrogenation of the acetate of the epidioxide (I).

Apart from hydrogenolysis, the structure of the epidioxide immediately admits of four reduction possibilities, viz., the three double bonds and the oxygen bridge. In acetic acid with

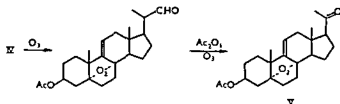
BARTON: That is a methyl ketone which is attached to a ring system, and one wouldn't argue that any sort of arithmetic of this type should be applied there. The hindrance there would seem to be mainly due to the hydrogen on the underneath side attached to C-14

REICHSTEIN: And C-12 of course.

BARTON: Yes, at C-12 as well.

SHOPPEE The argument that hindrance is due to C-14 α-hydrogen in the *C/D trans* series is substantiated by the fact that in the *C/D cis*-series both the rate and equilibrium relationships become inverted (Lardon, A., 1949, *Helv chim. Acta*, **32**, 1520).

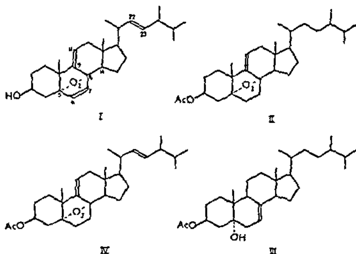
Degradation of the side chain of this dihydro-compound (IV) to the 20-ketone (V) has been accomplished by successive partial ozonolyses of (IV), and of the enol-acetate of the resulting aldehyde, the method first employed in the steroid series by Bergmann and Stevens (1948). The ozonolyses were best effected by adding a saturated solution of ozone in ethyl acetate to a similar solution of (IV) at $-70°$; the blue colour, initially rapidly discharged, persisting after the addition of approximately a molar proportion of the oxidant.



Having achieved suitable degradation of the side chain whilst preserving the 5 8-epidioxy-$\Delta^{9:11}$-system, attention was then directed to the problem of ring *C* oxygenation. This has been studied in a variety of ways, amongst which were the following.

Although isolated 9.11-double bonds are normally moderately reactive, the double bond in the system present in the tetrahydro-compound (II) and the 20-ketone (V) is distinctly unreactive towards oxidizing agents, presumably because of interference with the rear-wise approach of the reagent by the α-orientated dioxide ring. Only poor yields of a 9.11-epoxide were obtained with perbenzoic and peracetic acids, no reaction at all occurred with osmium tetroxide, and with permanganate in acetic acid some of a 9α:11α-glycol (yielding an 11α-acetate) was formed. With both permanganate and chromic acid there was a marked tendency towards allylic oxidation, leading to the formation of 12-keto-$\Delta^{9:11}$-compounds.

Adams catalyst the acetate of (I) rapidly takes up two moles of hydrogen, followed by two further moles at a much slower rate. Interruption at the half-way stage gave the tetrahydro-acetate (II), *isolated in 60 per cent yield, whilst the major* final hydrogenation product is 5α-hydroxyergost-7-enyl acetate (III), isomerized to the 8(14)-ene isomer on prolonged contact with platinum and acetic acid. Detailed investigations

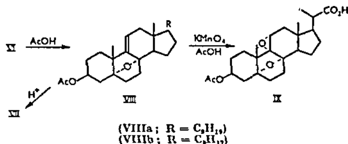


of the properties and reactions of the 5.8-epidioxy-$\Delta^{9\,11}$-system have been made with this tetrahydro-compound (II).

It seemed possible that the *cis*-6:7-double bond in (I) might be amenable to preferential reduction and many attempts were made to achieve this objective, working in acidic and neutral solutions with platinum, palladium and nickel catalysts, both with and without catalyst poisons. With platinum catalysts some of the required dihydro-acetate (IV) was eventually isolated from small-scale *experiments* by *careful* chromatography, and subsequently it was obtained in up to 60 per cent yields by employing platinic oxide trihydrate for the preparation of a platinum catalyst.

excess of this reagent dehydroergostery lacetate was re-formed from both the 5:8-diol (VI*b*) and the 7:9(11):22-triene (VII*b*).

Although the 5:8-diols readily give the conjugated dienes with mineral acids, with acetic acid even at room temperature they are smoothly converted into 5α:8α-epoxides (VIII). Their structures and stereochemistry follow from their smooth



(VIIIa; $R = C_9H_{19}$)
(VIIIb; $R = C_9H_{17}$)

isomerization under more strongly acidic conditions into the 5α-hydroxy-7:9-dienes (VII). The α-orientated epoxide ring in (VIII) offers much less hindrance to the rear-side approach of reagents; thus the 9:11-bond can be converted into an epoxide in 80 per cent yield with perbenzoic acid, and a 9α:11α-diol is formed with osmium tetroxide. With the 5:8-epoxide (VIII*b*), containing the 22:23-double bond, permanganate in acetic acid gives the 9:11-epoxide and, with more of the reagent, the acid (IX). These 5·8-9:11-diepoxides undergo several transformation reactions, but unfortunately these are apparently not straightforward and the precise nature of the changes which occur has yet to be elucidated.

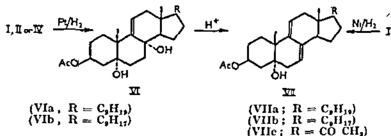
Acknowledgments

Grateful acknowledgment is made of the collaboration of Dr. P. Bladon, R B. Clayton, A. Crawshaw, C W. Greenhalgh, Dr. B. J. Lovell, Dr. G. Silverstone, G. W. Wood and Dr. G. F. Woods. The authors are indebted to Glaxo Laboratories Ltd for both financial support and for the preparation of intermediates by Dr. B. A. Hems and his colleagues.

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It has already been mentioned that further hydrogenation of the tetrahydro-compound (II) with platinum in acetic acid yielded mainly the 5α-hydroxy-Δ^7-compound (III), probably by dehydration of the 8-hydroxyl group, and then *partial hydrogenation of the 7:9-diene.* *Platinum-catalysed* hydrogenation of the tetrahydro-acetate (II) in concentrated ethyl acetate or dioxan solutions resulted in the separation of the sparingly soluble 5α:8α-diol (VI*a*), and similar experiments with the original epidioxide (I) and the dihydro-compound (IV) yielded a corresponding diol (VI*b*), still containing the 22:23-double bond. These diols are excessively



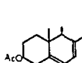
(VIa, R = C_9H_{19})
(VIb, R = C_9H_{17})

(VIIa; R = C_9H_{19})
(VIIb; R = C_9H_{17})
(VIIc; R = $CO\ CH_3$)

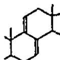
labile under acidic conditions, being transformed by mineral acids into the 5α-hydroxy-7:9(11)-dienes (VII). That containing the 22:23-double bond was first prepared by Windaus, Auhagen, Bergmann and Butte (1930) by the zinc-alkali reduction of (I). In our original studies of the partial hydrogenation of the epidioxide (I) it was found that such dienes were also produced when palladium or Raney nickel catalysts were employed, the use of the latter giving the triene (VII*b*) in 80 per cent yield. Reduction of the 20-ketone (V) with zinc and acetic acid analogously gave the 5α-hydroxy-diene (VII*c*) in the *allo*pregnane series and this has been converted into 11-ketoprogesterone.

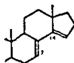
Attempts to effect anionotropic rearrangements of diols such as (VI) were precluded by their lability towards acids with the formation of the 7:9-diene system; phosphorus tribromide in dioxan gave the isomeric 8(9):14-diene. With an

diene (cisoid dienes) and yet have the two double bonds in different rings, e.g., a 7:14-diene (XII), then we find that photochemical oxida-

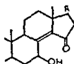


X

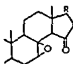

XI



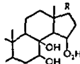
XII



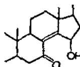
XIII



XIV



XV



XVI

tion proceeds smoothly. The products of reaction are XIII, XIV, XV and XVI We conclude therefore that it is the *cisoid* characteristic of the diene which is important rather than the energy required to excite the diene.

DISCUSSION

FIESER What is the essence of your evidence for the configuration at 5 and 8 in the 5 8-diol?

JONES The configuration of the 5-hydroxyl group was arrived at by consideration of its acetylation. It can be acetylated in exactly the same way as Plattner showed for other 5α-hydroxy groups by using acetyl chloride in dimethylaniline All the compounds that we have obtained with the 5-hydroxyl group can be acetylated under those conditions The 8-hydroxyl group must also have the α-configuration.

DJERASSI I might mention that we carried out a few experiments with this peroxide in the diosgenin series—we did not get very far because we turned to other series instead—but we tried to reduce it with $LiAlH_4$ and were unable to do so Have you tried this by any chance in the ergosterol series?

JONES Yes, it does work in the ergosterol series.

DJERASSI You get the diol?

JONES Dr. Barton, you did this didn't you?

BARTON From dehydroergosterol peroxide we prepared by $LiAlH_4$ reduction the same diol (3β 5α-dihydroxyergosta-7 9(11) 22-triene) as by zinc dust-alkali reduction. in the case of ergosterol peroxide itself, $LiAlH_4$ reduction affords the 3β 5α-dihydroxy-$\Delta^{7\,22}$ compound, the double bond migrating during the course of the reduction.

JONES Yours didn't reduce at all, Dr Djerassi?

DJERASSI No

BARTON Well it's a very slow reduction, one has to reflux overnight.

DJERASSI Ours was not done that long. It was refluxed in tetrahydrofuran for an hour or so, and the starting material was recovered; the UV spectrum and the infra-red were the same

COOK Prof Jones, have you considered the stereochemistry of the 5α:8α-oxide? It looks as though there ought to be a fair amount of strain

JONES We have made up molecular models, and there's very little strain associated with it The *B* ring is in the boat form and the 5 and the 8 positions come close together

BARTON I should like to say something about the mechanism of formation of the peroxide Prof Jones worked on The experimental facts are that if you take a diene system containing two double bonds in a six-membered ring (as X), and you dissolve this in alcohol solution with the addition of eosin, light, and oxygen, then you get the peroxide formed quite smoothly Prof Jones indicated his improvement in yield which makes this a very attractive reaction. It seemed to us that it might be possible to devise a photochemical method for the oxidation of the 7,9(11) steroidal diene system, XI, based on eosin, light and oxygen. If we take one of these systems or any of a number of others with transannular (*transoid*) double bonds, we find there's no reaction, or very very slow reaction— nothing like the type of reaction that occurs with a homoannular diene If, however, you take the other type of

Since the correct formulation of the side chain of ergosterol (cf. IV) as a disubstituted *trans*-ethylene (cf. Jones, 1950) requires a considerable amount of space, it is shown in the following figures in the old form as a disubstituted *cis*-ethylene.

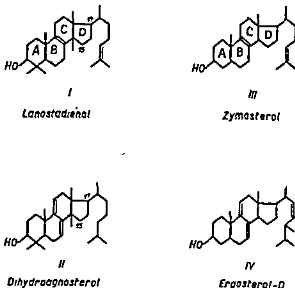


FIG. 1.

Before this close relationship between the steroids and the tetracyclic triterpenes of the lanostadienol type was known, it could be shown that the direct or stepwise oxidation of dihydroagnosterol acetate (II) and lanosterol acetate (V) led to the same unsaturated 1:4-diketone (VI) (Ruzicka, Rey and Muhr, 1944; cf. also Birchenough and McGhie, 1950, and Mijovic, Voser, Heusser and Jeger, 1952). The ditertiary double bond in the ene-1,4-dione (VI) could be reduced with zinc in glacial acetic acid solution. In the resulting diketone (VII) the two carbonyl groups exhibited a characteristic difference in reactivity. The one in ring *B* could be removed

INVESTIGATIONS ON THE SYNTHESIS OF 11-KETOSTEROIDS

H. HEUSSER and O. JEGER

THE recent work of Ruzicka, Jeger and co-workers (Ruzicka, Rey and Muhr, 1944; Voser, Montavon, Günthard, Jeger and Ruzicka, 1950; Voser, Mijović, Jeger and Ruzicka, 1951; Voser, Günthard, Jeger and Ruzicka, 1952; Voser, Jeger and Ruzicka, 1952; Mijović, Voser, Heusser and Jeger, 1952) and also the work of the schools under the leadership of McGhie and of Barton (Barnes, Barton, Fawcett, Knight, McGhie, Pradhan and Thomas, 1951; Barnes, Barton, Cole, Fawcett and Thomas, 1952; Barton, Fawcett and Thomas, 1951; Birchenough and McGhie, 1950; Cavalla and McGhie, 1951*a*; Cavalla and McGhie, 1951*b*; Cavalla, McGhie, Pickering and Rees, 1951; Cavalla, McGhie and Pradhan, 1951) have demonstrated that there exists a very close structural relationship between the steroids and the tetracyclic triterpenes lanostadienol (lanosterol) (I) and dihydroagnosterol (II).

In Fig. 1 these two triterpenes I and II are shown side by side with zymosterol (III) and ergosterol-D (IV). This unusual method of projecting the formulæ of lanostadienol (I) and dihydroagnosterol (II) has been employed in order to emphasize the close structural relationship between these two triterpenes and the steroids. The only point of uncertainty which still remains with regard to the constitution of compounds I and II is the precise point of attachment of the long side chain to ring *D*. This side chain could as well be attached to carbon atom 15 (numbered according to the steroid nomenclature) (Barnes, Barton, Cole, Fawcett and Thomas, 1952).*

*In the meantime the position of the long side chain has been proved to be definitely on carbon atom 17 (cf. I); Voser, W., Mijović, M. V., Heusser, H., Jeger, O. and Ruzicka, L. (1952). *Helv. chim. Acta*, 35, 2414.

It must be pointed out that there exists a close relationship between the results of other groups of workers and ours, in particular those of the Merck group (Chamberlin, Ruyle, Erickson, Chemerda, Aliminosa, Erickson, Sita and Tishler, 1951), the school of Fieser (Fieser, Herz and Huang, 1951; Fieser, Babcock, Herz, Huang and Schneider, 1951), the Syntex group (Stork, Romo, Rosenkranz and Djerassi, 1951; Djerassi, Mancera, Stork and Rosenkranz, 1951) and the school of Spring (Anderson, Budziarek, Newbold, Stevenson and Spring, 1951).

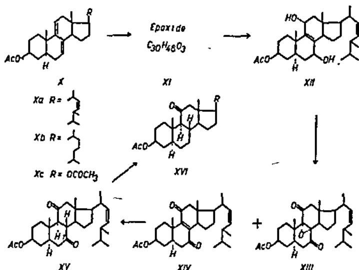


FIG. 3.

Experiments were carried out with 7:9(11)-steroid-dienes of the general formula (X), in which the substituent on carbon atom 17 was either the unsaturated side chain of ergosterol (X*a*), the saturated side chain of cholestane (X*b*) or an acetoxy group (X*c*). Similarly constituted compounds of the cholic acid series were also used as starting materials. In all cases the corresponding 11-ketosteroids (cf. XVI) could be obtained.

selectively by conversion to the mono-ethylene-dithioketal (VIII) followed by reductive desulphurization. By this means a ring *C* ketone was obtained (IX) which in all respects resembled an 11-ketosteroid (Voser, Montavon, Günthard,

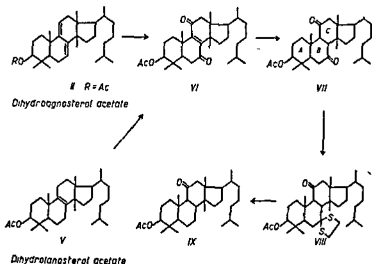


FIG. 2.

Jeger and Ruzicka, 1950; Voser, Mijović, Jeger and Ruzicka, 1951).

On the basis of these results, we have endeavoured to transpose this series of reactions to steroid-diene analogues with the object of finding a new method for the introduction of an oxygen-function in position 11.*

The following is a brief account of this research, most of the results of which have already been published (Heusser, Eichenberger, Kurath, Dällenbach and Jeger, 1951; Heusser, Heusler, Eichenberger, Honegger and Jeger, 1952; Heusser, Anliker, Eichenberger and Jeger, 1952*a*; Heusser, Anliker Eichenberger and Jeger, 1952*b*).

*In this work we have been generously supported by the Ciba Company in Basle and we would like to thank Dr. K. Miescher, Dr. A. Wettstein and Dr. G. Anner for their help.

For the following reasons we assume the epoxide from ergosterol-D acetate to be an 9:11-epoxide (XI). In absolute benzene solution it may be rearranged either with boron trifluoride or with anhydrous ferric chloride to the $\alpha{:}\beta$-unsaturated 11-ketone (XVII). This compound differs from the known isomeric $\alpha{:}\beta$-unsaturated 7-ketone (XVIII) (Stavely and Bollenback, 1943) in its melting point and its specific

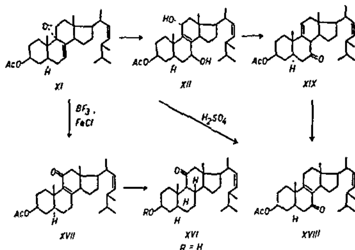


FIG. 4.

rotation. Both unsaturated ketones have practically identical UV-absorption spectra, with a maximum at 253 mμ. This is in agreement with the formulæ ascribed to them. The unsaturated 7-ketone (XVIII) arises from the same epoxide (XI) when this is treated with dilute sulphuric acid in dioxan, either by allowing the solution to stand for a long time or by warming it for a short time. We assume that the formation of the unsaturated 7-ketone (XVIII) from the epoxide (XI) *proceeds via the ene-diol (XII), since this compound could* also be converted stepwise via the $\beta{:}\gamma$-unsaturated ketone (XIX) to the α,β-unsaturated 7-ketone. Tishler (Schoenewaldt, Turnbull, Chamberlin, Reinhold, Erickson, Ruyle,

In the following, however, we shall only consider the work done on the ergosterol series. Ergosterol-D acetate (X*a*) can easily be obtained in high yield by the hydrogenation of ergosterol acetate in ether with Raney nickel, followed by the dehydrogenation of the resulting 5-dihydroergosterol acetate with mercuric acetate. The direct energetic oxidation of ergosterol-D acetate (X*a*) under the conditions used for triterpenes did indeed lead to the required unsaturated diketone (XIV), but the yields obtained were not very satisfactory for preparative purposes (cf. also Djerassi, Batres, Velasco and Rosenkranz, 1952). Better results were obtained by the stepwise oxidation of ergosterol-D acetate (X*a*). A good yield of an epoxide (XI) whose constitution must be left open for discussion, was obtained by the controlled oxidation of ergosterol-D acetate (X*a*) with mono-perphthalic acid (cf. Chamberlin *et al.*, 1951; Anderson, *et al.*, 1951). Mild hydrolysis of this epoxide with simultaneous allylic rearrangement led to an 8(9)-unsaturated 7:11-dihydroxy steroid (XII). Oxidation of this ene-1:4-glycol with an excess of chromium trioxide gave a diketo-ditertiary epoxide (XIII) as the main product. Smaller quantities of the ene-1:4-dione (XIV) could also be isolated from the reaction mixture (cf. also Chamberlin *et al.*, 1951; Fieser, Babcock, Herz, Huang and Schneider, 1951). Both compounds, the ditertiary epoxide (XIII) and the ene-dione (XIV), could be reduced with zinc in glacial acetic acid to the saturated 7:11-diketone (XV) in almost quantitative yield. This compound (XV) has the same configuration as the naturally occurring steroids at the points of fusion of the rings *B* and *C*. The selective reduction of the keto group in position 7 of the diketone (XV) was achieved by converting it either to the corresponding 7-mono-ethylene-dithioketal followed by reductive desulphurization, or by a direct Wolff-Kishner reduction using the modification of Huang-Minlon (1946). Further work was directed towards proving the constitution of the epoxide (XI). Other variations of the synthesis were developed, which led to 11α-hydroxysteroids in very good yield.

trifluoride in benzene. The elimination of the tertiary hydroxyl group in the keto-glycol (XXII) proceeds exceptionally well in a two-phase system with sodium hydroxide solution and dioxan. The α:β-unsaturated γ-hydroxy-ketone (XXIII) obtained yields a diacetate. Both (XXII) and (XXIII) were first described by Spring and co-workers (Anderson, Budziarek, Newbold, Stevenson and Spring, 1951), who obtained (XXII) from ergosterol-D acetate epoxide as follows: The double bond in the side chain was first protected by bromine, the crude bromination product was treated with an excess of perbenzoic acid and finally, without isolating any intermediate products, a debromination with zinc in glacial acetic acid was carried out. We believe that in this case too, the keto-glycol (XXII) is formed via intermediate products of the type (XII) and (XX).

Finally, the ditertiary double bond in the α:β-unsaturated γ-hydroxy-ketone (XXIII) may easily be subjected to selective hydrogenation. The 3β:11α-dihydroxy-7-ketoergost-22-ene (XXIV) is thereby obtained; this gives a diacetate. The removal of the keto group on $C_{(7)}$ in compound (XXIV) may again be easily accomplished by a Wolff-Kishner reduction. In this way we obtained the desired end product of this series of reactions, namely 3β:11α-dihydroxyergost-22-ene (XXV), which was characterized by its diacetate and also by its oxidation to the corresponding 3:11-diketone (XXVI); (XXV) is also obtained in high yield by the reduction of the 11-ketone (XVI) with sodium in propanol.

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BARTON, D. H. R., FAWCETT, J. S., and THOMAS, B. R. (1951). *J. chem. Soc.*, 3147.

Chemerda and Tishler, 1952) and also Rosenkranz and Djerassi (Sondheimer, Yashin, Rosenkranz and Djerassi, 1952) have recently proved the correct interpretation of our results by the reduction of the α:β-unsaturated 11-ketone (XVII) with lithium in liquid ammonia to the corresponding 11-ketoergost-22-en-3β-ol (XVI).

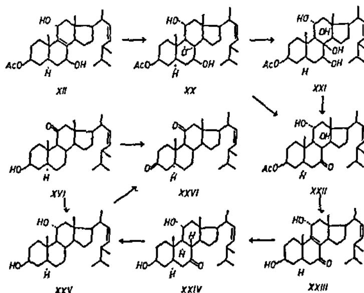


FIG. 5.

The ene-diol (XII), which can be prepared from the ergosterol-D acetate epoxide (XI) in almost quantitative yield, gives an epoxide (XX) in which both hydroxy groups on $C_{(7)}$ and $C_{(11)}$ can be acetylated. Mild hydrolysis of the epoxide (XX) with sulphuric acid leads to a tetrol (XXI) which splits off water on energetic treatment with sulphuric acid or under the influence of hydrogen bromide to give the keto-glycol (XXII). This keto-glycol (XXII) is also obtainable in particularly good yield through the isomerization of the epoxide (XX) with hydrogen bromide in glacial acetic acid or boron

DISCUSSION

BARTON: I was interested in the configuration in your tetrol XXI at C-7, which you formulate as 7β.

HEUSSER: And in the enediol (XII)

BARTON: And in the enediol, too. On the simple basis one might expect it to be 7α. I know your arguments against that—I think they're very good arguments—but before I saw them I thought the [illegible]

FIESER. In the work that Hans Heymann did in my laboratory recently we had a 9 11 glycol, and our evidence showed that the dehydration of that proceeded by a *cis* rather than a *trans* elimination. We interpreted it as a neighbouring group effect, so that it seems to me possible that the same thing could happen in your case.

DJERASSI Was that under acid conditions?

FIESER: Yes.

HEUSSER But you used very strong conditions for the formation of the ketone I believe you used $SOCl_2$ or HBr, and *in our case elimination* goes under very mild conditions—only allowing the substance to stand in acetic acid with a few drops of dilute sulphuric acid.

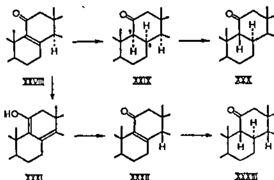
FIESER I don't remember our conditions, but I don't think that they were terribly drastic We were expecting to get *trans* elimination, and

glycol

BARTON You must have the *trans*; and yet this *trans*-glycol dehydrates very easily

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CHA ... A. F., CHEMERDA, J. ... FISHLER
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SCHOENEWALDT, E., TURNBULL, L, CHAMBERLIN, E. M, REINHOLD D, ERICKSON, A. E, RUYLE, W. V., CHEMERDA, J. M., and ... M. (1952) *J Amer. chem Soc.*, 74, 2696
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One of the troubles about the chemical reduction of this type of compound is that as soon as you form an enol from it with a double bond at 8(14) (XXXI), the *trans C/D* junction may be converted to the



XXXII; presumably if it proceeds in the same way here as when the *C/D* junction is *trans*, it will give the correct configuration at 8 and 9 but inverted at 14. One can then build up various 11-oxygenated

that reaction?

HEUSSER No. We also carried out reductions like this in liquid ammonia, but when we did the reduction with sodium in alcohol then

HEUSSER We don't know.

DJERASSI: The *C/D* juncture would be either *trans* or *cis*; but in addition you could have three abnormal isomers at *B/C*.

HEUSSER. Inverse configuration at C-14, C-9 or C-8 (XXXIV, XXXV, XXXVI, p. 58)

DJERASSI: The way we look at it is this; if you get *cis* addition of hydrogen at *B/C*—and that was primarily our argument which Dr.

order to preserve the Walden inversion at every centre you must finish up with 7α.

HEUSSER: Or we can also believe that from C-9.

BARTON: It depends on the lifetime of the intermediate carbonium ion.

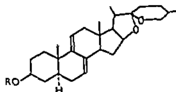
a mono-epoxide, wh

by Dr. Heusser, or

This compound, wh

BF_3 in benzene to a

evidence is the $\Delta^{8(9)}$-11-ketone: when it was treated with lithium in liquid ammonia it could be reduced very smoothly to 11-ketotigogenin. I think this is conclusive proof that the original ketone had been



XXVII

the $\Delta^{8(9)}$-11-ketone and hence that the oxide had been the 9:11 epoxide. If it had been the 7:8 epoxide it would have given the $\Delta^{8(9)}$-7-ketone. I think it is of interest that when the above-mentioned reduction is done in the presence of alcohol and excess lithium, 11α-hydroxytigogenin is formed. This reaction seems to proceed via the 11-ketone, which under the reaction conditions employed is reduced to the 11α-ol. I might just mention that 11-ketotigogenin has already been converted to cortisone, so that the reactions just described complete another

reochemistry, I think the following
what we now know is the $\Delta^{8(9)}$-11-
atalytically with hydrogen over palladium, it takes up one mole of hydrogen and gives an isomer of 11-ketotigogenin which cannot be converted into the latter by alkali. *Cis*-addition of hydrogen presumably takes place from underneath the molecule to give the 8α 9α compound XXIX which, having a ketone

PARTIAL SYNTHESIS OF $\Delta^{9(11)}$-ANHYDROCORTICOSTERONE ACETATE

R. CASANOVA, A. RUFF and C. W. SHOPPEE

Dehydration of corticosterone acetate (Shoppee and Reichstein, 1943) led by ionic 1:2-elimination to an anhydrocorticosterone acetate (I) which exhibited high activity in regard to life maintenance and mineral metabolism. This substance, m.p. 159°, $[\alpha]_D + 129°$, was later found to be different from Δ^{11}-anhydrocorticosterone acetate (II), m.p 180°, $[\alpha]_D$ +178° (Meystre and Wettstein, 1948; Euw and Reichstein, 1948), which also exhibited high life maintenance activity. These two substances appear to be of interest in connexion with possible mechanisms of biochemical oxygenation at C-11.

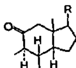
It seemed desirable to verify the $\Delta^{9(11)}$-formulation (I) and a partial synthesis was attempted from deoxycholic acid (III), at that time the only available starting material. Dr. Ruff found that it was impossible to apply the bromination stage of the Miescher-Wettstein degradation to 3α-hydroxychol-9(11)-enic acid derivatives; the desired partial synthesis has been achieved by way of ætiodeoxycholic acid (IV) and 3α-hydroxyetia-9(11)-enic acid (V) by Dr. Casanova:

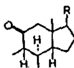
Wolff-Kishner reduction of the methyl ester (V) was unsatisfactory, but use of the thioketal-nickel procedure gave the ester (VI) [m.p. 132°, $[\alpha]_D + 69°$] oxidized by *N*-bromoacetamide to the keto ester (VII) [m.p. 125–126°, $[\alpha]_D + 56°$] (Lardon and Reichstein, 1945). Bromination in acetic acid furnished the 4ξ-bromketone (VIII) which was unaltered by treatment with pyridine at 100–120° but was converted by the method of Mattox and Kendall into methyl-3-keto-4:9(11)-dienate (IX) [m.p. 104–105°, $[\alpha]_D + 132°$].

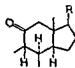
The acid corresponding to the ester (IX) was obtained in

Sondheimer mentioned—you would not expect addition of hydrogen [illegible] methyl group, because that side is [illegible] if you get the opposite *cis* ad- [illegible] isomer, which would be quite [illegible] *xperientia*, 8, 315). [illegible] isomers of yours, Dr. Heusser, [illegible]

DJERASSI: You asked, Prof. Jones, whether in the reduction of the Δ^{8}-7-ketone, you invariably get directly the *trans* configuration. We [illegible] isolate a *cis* intermediate and to see whether the [illegible]







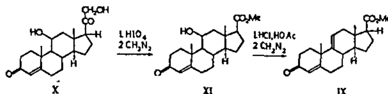
XXXIV XXXV XXXVI

[illegible] And that [illegible]

be more stable [illegible] 1-boat 2-chair conformation and the other [illegible] 1-chair conformation. It depends on whose models you're using.

SONDHEIMER: I quite agree with you, Dr Barton. All we can say is that this compound is *either* the inverted compound (8α 9β) or else the 8α:9α, if that configuration is the more stable. Which of the two configurations appears the more stable depends, as you say, on what models one uses That's why I say we have only very provisionally assigned it the 8α·9β structure.

furnished the doubly unsaturated ester (IX), which was identical with the product obtained by partial synthesis from (III). The ester (IX) has also been obtained from I as the free alcohol by degradation with periodic acid.



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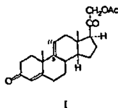
DISCUSSION

...mide with
pyridine
we used
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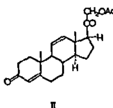
dibromo-5α-steroid and collidine, if the collidine contains much water the dehydrobromination is inhibited completely. If a little water is added—and the amount is crucial—the 4-bromine will be eliminated, the 2-bromine will not be, and one can obtain a reasonably good yield of the 2-bromo-Δ^4-3-ketone. If completely anhydrous collidine is used —and for that one really has to fractionate with a 30-plate column—one can eliminate both bromine atoms in perhaps one-tenth the time required ordinarily.

Regarding your discussion about the configuration of the bromine. I did come to the same conclusion at one time, but I think Norman Jones has come to the opposite conclusion on the basis of infra-red spectra, in that he thinks that all the 4-bromo 5β steroids that he has

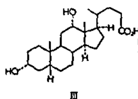
the first instance in quantity insufficient to permit its conversion with lithium methyl into $\Delta^{9(11)}$-progesterone and by the diazo-ketone synthesis into $\Delta^{9(11)}$-anhydrocorticosterone



I

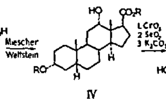


II



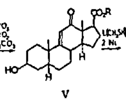
III

Deoxycholic Acid

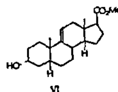


IV

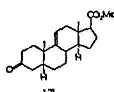
R = cathyl or methyl succinoyl



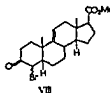
V



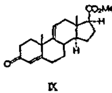
VI



VII



VIII



IX

acetate (I). In the meantime, therefore, corticosterone (X) has been degraded with periodic acid and subsequent treatment with diazomethane to the 11β-hydroxy-ester (XI), which by dehydration with hydrochloric acid-acetic acid

DJERASSI: Usually the collidine hydrobromide precipitates almost immediately.

REICHSTEIN: I just wanted to ask Prof. Shoppee what the $\Delta^{9(11)}$ bond does in the bromination process, when he treats VII with bromine in acetic acid to get the crystalline monobromide. This double bond at 9(11) is very susceptible to bromination, and afterwards elimination of HBr. Something must occur here as a side reaction

SHOPPEE: I can only say that Dr. Ruff made a lot of experiments on $\Delta^{9(11)}$ compounds with bromine, as did Kendall. We found that in this [illegible]

SHOPPEE: Ultra-violet.

H. HIRSCHMANN: We had an opportunity to measure the infra-red spectrum of a sample of methyl 3α-acetoxy-eti-9(11)-enate which we got from Dr. Prins. I shall show the curve in the afternoon I feel it agrees well with those of other $\Delta^{9(11)}$ unsaturated compounds in the 12 μ region.

PINCUS: As one of the biologists here, I would like to ask about the problem of activity. I wonder if more could be said of the fact that in the Everse-de Fremery test this $\Delta^{9(11)}$ material appeared to be more active than 11-deoxycorticosterone. I was a little puzzled about the life maintenance test

SHOPPEE: In the life maintenance test in rats it showed slightly less or approximately equal activity. In the Everse-de Fremery test it showed 2–3 times the activity of DCA. Why there should be this difference I don't know.

MARRIAN: I would suggest to Prof Shoppee that he give a minute amount of this material to Mrs Simpson for a test of mineralo-corticoid activity.

SHOPPEE: [illegible]

MARRIAN: [illegible]

SHOPPEE: [illegible]
little of the [illegible]

PINCUS: [illegible]
cortisone) derivatives have ever been prepared.

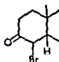
SHOPPEE: No, they've never been prepared. I'm working on it now.

DJERASSI: I know that Dr. Fieser has one of the best pieces of evidence concerning the configuration of the 4-bromine atoms in a 4-bromo 5β-compound (XII).

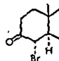
FIESER. Our evidence is that if you reduce the 3-keto group with sodium borohydride you get a mixture of the two epimers at 3, which are separable (XIII and XIV). One of them on dehydrobromination [illegible]

examined by infra-red spectroscopy have the 4β configuration XII, in other words, they would all be *cis*, provided ring *A* is in the chair conformation. The same thing holds true for the 5α-compounds, in

(*J. Amer. chem. Soc.*, 1949, 71, 1005)—and yet on the basis of infra-red



XII



XIIA

spectra Jones suggested that the 4-bromine would have the α configuration. Of course these compounds could invert instantaneously at C-4 in hot collidine or pyridine, since the bromine is on a carbon adjacent to a keto group (C-3). So probably elimination evidence cannot be used with absolute certainty.

SHOPPEE: Yes, Dr. Djerassi, I saw this paper by Norman Jones, but I haven't had time to study it. In regard to Dr Sarett's question about the dryness of the pyridine, our pyridine is normally prepared by refluxing over barium oxide and then distilling in a vacuum in a stream of nitrogen. It may be rather dry—but you don't know how much water it picks up when it's being transferred.

SARETT. That's not enough.

DJERASSI. Have you any explanation for this?

SARETT. No.

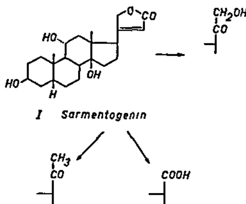
REICHSTEIN. Since you spoke about the elimination of HBr from bromoketones it might be appropriate to mention cholest-1-en-3-one. One of my students prepared the well known 2-bromocholestanone and

REICHSTEIN: There was no water in this. When you distil the collidine very carefully all the water comes out. This was really very pure collidine.

CORTISONE AND 11-EPI-17-HYDROXYCORTICOSTERONE DIACETATE FROM SARMENTOGENIN

A. LARDON and T. REICHSTEIN

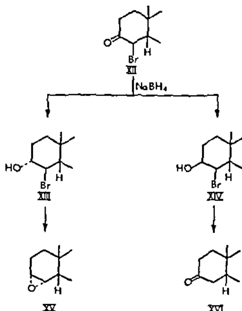
Of all natural products sarmentogenin (I) is certainly one of the easiest to transform into cortisone. It already contains a hydroxyl group in the 11-position, and the lactone side chain can easily be degraded to the ketol, methylketone or carboxyl group. One disadvantage is its difficult accessibility.



I Sarmentogenin

So far sarmentogenin has been obtained mainly from different *Strophanthus* seeds (Jacobs and Heidelberger, 1929; Tschesche and Bohle, 1936; Euw and Reichstein, 1950*a*, 1950*b*; Euw, Reber and Reichstein, 1951; Rothrock, Howe, Florey and Tishler, 1950, Callow, Meikle and Taylor, 1951). Recently it has also been found in the bulbs of *Rhodea japonica* Roth (liliaceæ) (Nawa, 1951, 1952). It was first isolated by Jacobs and Heidelberger (1929) from *Strophanthus* seeds of

3-ketone (the 4-bromo-3-ketone) has the β-orientation We have also applied it in the 5α-series to cholestan-3-one; the 2-bromocholestanone obtained in high yield on bromination is the 2β-bromo compound.



BARTON: As most of the brominations are carried out in acetic acid in the presence of HBr we have to be certain that the product we *isolate* is really the kinetically controlled product of bromination and not the thermodynamically controlled product which has been formed by isomerization. In a case studied earlier in the literature (Barr, Heilbron, [illegible]

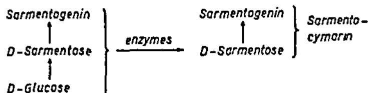
[illegible]

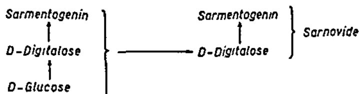
you get the same isomers as you do with bromine in acetic acid. And in fact you have no HBr present.

BARTON: Which ketones do you mean?

DJERASSI Cholestanone, coprostanone and a number of related 5α- and 5β-3-ketones. In each case we obtained the same isomers whether we used N-bromosuccinimide in carbon tetrachloride or bromine in acetic acid. This work was published in *Experientia*, 1947, 3, 107.

Jacobs and others). In the seeds neither sarmentocymarin nor sarnovide is present as such in detectable quantities but in the form of di- or triglycosides, probably bound to one or two molecules of glucose.





Sargenoside

If defatted seed powder is extracted with alcohol directly all enzymic degradation is prevented. From such extracts no sarmentocymarin or sarnovide was obtained. But after acetylation it is possible to isolate a crystalline hexacetate of a diglycoside which we now call sargenoside. (The hexacetate was originally called sarmentoside-*B*-acetate.) Callow and Taylor, as well as we, found independently that the corresponding free diglycoside (not known yet in pure form) is composed of sarmentogenin, digitalose and D-glucose.

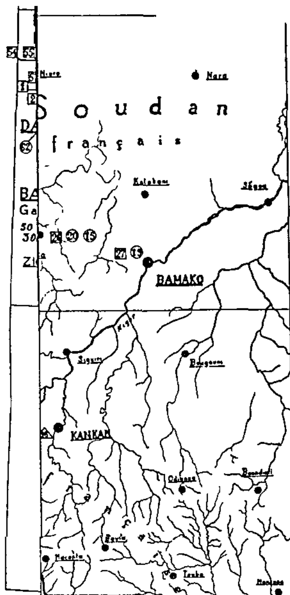
If on the other hand the water-soluble part of the enzymes contained in the seeds is allowed to act, no sargenoside hexacetate is obtained but crystalline sarmentocymarin and sarnovide. The latter apparently is formed from sargenoside.

doubtful origin; they had good reasons to assume that the material was *S. sarmentosus*, and that was the reason for the name "sarmentogenin". *S. sarmentosus* is distributed over a vast area, covering the greater part of western tropical Africa.

In 1948 we investigated about a dozen different samples of authentic *S. sarmentosus* from the eastern part of the Ivory Coast, Togo, Gold Coast and Southern Nigeria. After fermentation we found that they contained no sarmentocymarin ($C_{30}H_{46}O_8$), or only traces, but another glycoside which we named sarveroside ($C_{30}H_{44}O_{10}$) (Euw, Katz, Schmutz and Reichstein, 1949; Buzas, Euw and Reichstein, 1950).

Small amounts of sarmentocymarin were found in *S. petersianus* (Euw and Reichstein, 1950*a*) and *S. Courmontii* (Euw and Reichstein, 1950*b*). By the courtesy of Prof. Th. Monod and Dr. A. Pitot we obtained in 1950 a large sample of seeds of the variety of *S. sarmentosus* growing in French Senegal. These seeds were chemically very different from those mentioned above. After fermentation they gave a high yield (0·34 per cent) of sarmentocymarin and some 0·1 per cent of a new glycoside which we called sarnovide and which was the D-digitaloside of sarmentogenin. No trace of sarveroside was obtained. I shall show you later a few pictures of this sarmentogenin-producing variety, which we provisionally gave the No. *MPD* 50. The flowers are practically identical with the plants from Togo, etc. There are small differences in the shape of the leaves and big differences in that of the fruits.

Plants which gave the same chemical results as our No. *MPD* 50 were found independently by Callow, Meikle and Taylor (1951) in Northern Nigeria. I shall later say a few words on the plants growing in the area in between. Dr. Callow kindly accepted an exchange of seed material. It was found that originally apparent small differences in results were only due to use of different analytical methods. We analyse *Strophanthus* seeds after enzymic degradation of the crude glycoside mixture (using methods developed by



ollected.

The di- or triglycoside which yields sarmentocymarin in the fermentation process has not yet been isolated and no real attempts were undertaken in this direction. We prefer to include the fermentation step in the analysis and to isolate the monglycosides, which are much easier to separate.

Distribution of the Sarmentogenin Producing Strain of *S. Sarmentosus*

Owing to very generous financial help by Ciba (Basel) and Organon (Oss) two of my co-workers were able to participate in an expedition in the spring of 1951, when a great number of different seed samples were collected. Among these were more than 50 samples of individual single plants: this is important to make sure of homogenous material. The map (Fig. 1) shows a number of locations where seeds were collected. You see that the region covers important parts of Senegal, Sudan and a border line of French Guinea. We also had about 20 samples from further south, mainly collected by the French botanist Dr. Schnell. All the samples were analysed in Basel and only a very few are not yet finished. We are therefore quite well informed about the western part of the distribution area. The northern limit is the desert, while the southern limit of the sarmentogenin-producing variety is roughly 12° latitude. *S. sarmentosus* grows abundantly further south, but there the plants contain no sarmentocymarin, or only traces. We are not well informed on the region further east. But as Callow and Meikle found the same variety in Northern Nigeria it is quite possible that the zone where it grows includes parts in the north of Togo and Gold Coast and stretches from the Atlantic as far as Lake Chad.

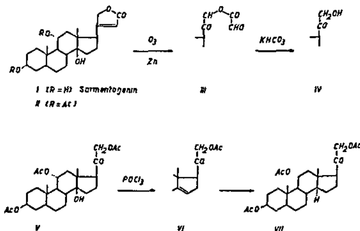
Plants which the botanists classify as belonging to the some species, *S. sarmentosus*, are found as mentioned in a much bigger area covering the whole western tropical part of Africa through the Cameroons, Gabon, Belgian Congo, as far as North Angola. The plants growing between the Cameroons and North Angola are recognized as a distinct variety *S. sarmentosus var. major* Dewèvre. This produces

sarveroside and panstroside but no sarmentocymarin. In other words, it gives the same results as the plants between the eastern part of the Ivory Coast and Southern Nigeria. If one were allowed to speak of chemical varieties, a third one would have to be distinguished which grows in French Guinea and the western part of the Ivory Coast. This one is very poor, really almost devoid of glycosides, and contains only traces of intermedioside and panstroside. It is quite possible and even probable that on the borderlines between the three big areas mentioned, mixed forms can be found. A few results pointing in this direction were obtained in earlier work. But in the analysis of seeds derived from *single* plants we were astonished to find no real indication of mixed forms. The samples contained either sarmentocymarin or sarveroside, but not both. This may be chance or partly the result of the fact that the seed samples collected from individual plants were usually small, and by-products would not have been found if present merely in traces On the other hand, sometimes in an area where practically only sarmentogenin-producing plants grew, single ones were sometimes found which contained only sarveroside. If mixed seeds from a great number of plants of such an area were analysed, the result would have given the impression of mixed forms.

As a curiosity I may add that among the many individual plants we found two which produced as main glycosides two new compounds which we named "sarmutoside" and "musaroside." These contain a new aglycone, sarmutogenin, linked again to the same two sugars D-sarmentose and D-digitalose, as found in the other two pairs of glyconides (sarmentocymarin-sarnovide and sarveroside-panstroside). The formula of sarmutogenin is $C_{23}H_{32}O_6$, between sarmentogenin $C_{23}H_{34}O_5$ and sarverogenin $C_{23}H_{32}O_7$.

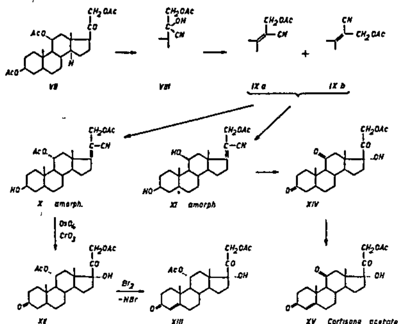
Another distinct variety is *S. sarmentosus var. glabriflorus*
We owe a small seed sample
These seeds were extremely
ere of the type found in *S.*

The best sample of the sarmentogenin-producing variety of *S. sarmentosus* gave 0·78 per cent sarmentogenin and about 0·4 per cent sarnovide; i.e. 1·18 per cent of sarmentogenin glycosides altogether. This and a few other selected seed samples are being tested for experimental cultivation, but results cannot be obtained for 3-4 years. It is of course very doubtful whether *Strophanthus* seeds with high sarmentogenin content may ever be produced cheaply enough to be of practical use as a source for cortisone. But in order to show that the chemical transformation is really possible and easy, I show in the following diagram some of Dr. Lardon's results.



It is just one of the many possible ways. Starting with sarmentogenin diacetate (II) it is not necessary to isolate the intermediates III, IV, V and VI. If carried out in this way the crystalline triacetoxyketone (VII, m.p. 178°) was obtained in 31 per cent yield. Dr. Callow and Dr. Taylor have informed me that they have prepared this ketone too in a similar way.

The biggest loss probably occurred in the hydrogenation step (VI-VII) where two stereoisomers are formed. It should probably be possible to increase the yield appreciably. Instead of VII other useful intermediates can be produced from sarmentogenin.



For the transformation of VII into cortisone and 11-epi-17-hydroxycorticosterone diacetate (XIII) we used the cyanohydrin process (Sarett, 1949) as shown in the diagram above, again without trying to adapt it to this particular case After addition of HCN and elimination of water a mixture was obtained which we think is composed of the two *cis-trans*-isomeric cyanohydrins IX*a* (m.p. 150°) and IX*b*. One of these was obtained in crystals. But the mother liquor gave practically the same results as the crystals in the subsequent reactions; this is the reason why we think it contained a

stereoisomer. Alkaline hydrolysis and partial acetylation in the 21-position gave a mixture of the diacetate X and the monoacetate XI which could be separated by chromatography. These two compounds after hydroxylation with OsO_4 and dehydrogenation with CrO_3 gave the diketone XII (m.p. 222°) and the triketone XIV (m.p. 226°) respectively, both well crystalline. The latter was known already. By bromination and dehydrobromination XIII (m.p. 201°) and XV were obtained. The last one was identical with material from adrenal glands.

The yields in the transformation of VII into XII or XIV were not satisfactory at all. So far no steps have been undertaken to work out these reactions or to find a better route. As mentioned, Sarett's method was used just as described. One reason for the low yield may be that the compound VII is not very suitable for this process. Sarett used it originally for a compound which contained only one nuclear acetoxy group in the 3α-position, which is very easy to saponify. The two acetoxy groups of VII are in the 3β- and 11α-positions and are both rather difficult to hydrolyse. In the saponification of IX*a* and IX*b* relatively drastic conditions had to be used and the cyano-group may have been partly attacked.

(Pictures of *Strophanthus sarmentosus* and related species were then displayed; cf. coloured plates in recent issues of *Helv. chim. Acta.*)

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DISCUSSION

CALLOW: Prof Reichstein's pictures took my memories back to Nigeria. I have been particularly interested in the problem of just what

[illegible]

available, so he stopped that work. But to come back to this species difference—it does look as if there is some chance of there being a chemical species

REICHSTEIN. I quite agree with Mr. Meikle that there may also be morphologically intermediate forms, but it is queer that when we investigated seeds derived from single plants, we could only in a very few cases get one which contained both sarmentocymarin and sarverosidе Such samples nearly always gave *either* one *or* the other. Regarding the question of practical use I never had the opinion that collection of wild seeds could serve for such a purpose. They could only be useful if selected plants were cultivated on a sufficiently large scale.

CALLOW· That is our experience too.*

REICHSTEIN *The analyses of seed* derived from single plants were done with very small quantities But if you take a big batch which is derived from very many plants of one type, then you can nearly always

**Note added* 1 Dec.52. Dr. Bush and Dr. Taylor now find both substances by chromatographic methods in our collections from single plants

trace small amounts of the other. The reason for this may be either the more accurate analysis or the mixture of seeds. In a limited area where, say, sarverogenin-producing plants occur, you can sometimes find odd plants which contain only sarmentogenin, and *vice versa*. If you mix all the seeds together and analyse them afterwards the result would be wrongly interpreted as being due to an intermediate form. But even if the analysis of seeds from individual plants has so far given no chemical evidence for intermediate forms I have never denied that such forms may exist. On the contrary, I am convinced that they must exist, because the plants are so nearly related to each other that they will hybridize. For instance, in a certain region of Senegal where only the sarmentogenin plants (with slim fruit and thin walls) were growing we found two plants which contained much sarverogenin;

They are only reluctant to recognize them as definite varieties because there are these intermediate forms. I think some botanical difference will be found if the two pure forms can be grown on the same spot. We are trying this now; we have sent some seed material to different places in Africa and America where they are being grown. If these experiments are successful, when the seeds appear for analysis one can see whether the chemical differences are only due to conditions of atmosphere and soil or whether these are genetic differences.

CALLOW. Seeds that we brought back have been germinated and are growing in one of the hothouses at Kew, and there is the characteristic

PINCUS: Are you convinced that the enzymic activity is really giving you optimal yields?

REICHSTEIN: I'm not quite convinced about that, but we didn't find great loss. I think Dr. Callow has found one point where a certain loss may occur—the purification of the crude extracts with lead hydroxide.

enzymes from *Strophanthus* seeds and use such preparations, but the activity seems to be partly destroyed during the isolation process.

HECHTER: Is there any evidence for plant enzymes which might introduce the 11α-hydroxyl group?

REICHSTEIN: We haven't tried that. I think there must be some enzyme formed in the plant somewhere; whether it's in the ripe seed, I don't know.

DORFMAN: Is there a significant concentration of cardiac glycosides in other parts of the plant?

REICHSTEIN: It is very low, and the by-product predominates. It is usually more satisfactory to analyse 20 g of ripe seed than 5 kg. of wood of the same plant. We have tried, for instance, with *Strophanthus gracilis*.* In this case it was possible to get sufficient seed material for paper chromatography but not for preparative isolation. We therefore did the preparative separation from the wood, which took about 2 years instead of the usual 4 weeks for an analysis of a seed sample. Moreover the glycosides in the wood of *S. gracilis* are different from those of the seeds of the same species; as far as could be judged from paper chromatography of the seed sample, there are quantitatively great differences and also qualitative differences. Similar observations have been made long ago with other plants. The seeds in *Digitalis purpurea*, for instance, contain different glycosides from the leaves. I had the impression (I think that's more or less gone now with these

PINCUS: Does *Strophanthus* have to grow in a tropical climate?

*Regarding the analysis of *Strophanthus* seeds I could add the following

REICHSTEIN: Yes.

PINCUS: How many years does it take to flower normally?

CALLOW: I don't know definitely—2–5 years. As to the age of these plants one could only make a rough guess. Once we found a *S. sarmentosus* being cultivated for its arrow poison, with stems 40–50 cm. in circumference. We asked an old chap, "How old is that?" After a lot of talking, he said his father planted it—"It's probably 100 years old, master."

PINCUS: Well that should be good if it flowers every year.

CALLOW. They flower profusely, as Prof. Reichstein's pictures have shown But I've collected the seed crop from the previous year off a bush flowering profusely, and it amounted to 18 pairs of seed pods. The fertility is extremely low.

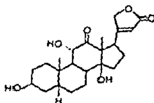
REICHSTEIN I once sent a few seeds of *Strophanthus Preussii* to one of my friends in Africa, a farmer. He planted them, and after three years the first flowers appeared. He pollinated them artificially and at the end of the same year he got 7 fruit. It wasn't much use, for the species is very poor in glycosides My co-worker, Dr. Speiser, reported that in Senegal and French Soudan plants of *S. sarmentosus* from 50 to 100 years old in a good situation produced between 50 and 150 fruit each Others produced only a few fruit because they were severely damaged by fire or for other reasons. My impression was that you could get the same yield in seed from *S. sarmentosus* as cotton fibre from *Gossypium* in kg per acre. If grown under appropriate conditions it should therefore be possible to produce *Strophanthus* seeds at roughly the same price as crude cotton fibre. (*Gossypium* seeds contain about 30 per cent useful fibre)

PINCUS. That's a very good yield. Maybe they can also use these lovely glistening things which come off the seed What do you call them?

CALLOW. Plumes or comose appendages.

KLYNE: Is anything known about the position of the "extra" oxygen atoms in sarmentogenin and sarverogenin?

REICHSTEIN. Perhaps Dr Callow may know a little more about sarverogenin. We have not done many experiments with this compound. In sarmutogenin, I think that the sixth oxygen is present as a keto group at C-12 (XVI) but it could also be an 11-keto-12-hydroxy derivative.



XVI

[illegible]

enough to assume that these two compounds may differ also in the substituents in ring C.

As already mentioned, sarmutogenin has so far been found in only two single plants. These may be mutants but they may also represent two odd plants of a variety or a strain of *S sarmentosus* which has its main distribution in another area, not yet discovered Oxidation of sarmutogenin with chromic acid gives the triketone sarmutogenone. The same triketone was obtained from another genin, isomeric with sarmutogenin, found in some Asiatic *Strophanthus* species. The distribution of oxygen atoms must therefore be the same as in sarmutogenin. The difference may be due either to stereoisomerism, or the keto and [illegible]

[illegible]

hindered?

CARDWELL: The 7-keto bears practically the same relationship to the 14-hydroxyl group that the 12-keto does in digoxigenone, and the [illegible]

[illegible]

reason to support the assumption that it's a hindered position?

BARTON: Not if it's a 14β compound. If it were 14α I would agree that it ought to be hindered at C-7. If it's 14β I'm a little surprised. Does this 7-ketone oximate with pyridine and hydroxylamine hydrochloride?

REICHSTEIN We didn't try it with pyridine And we didn't try it with sarverogenin, but sarmutogenin does not react on boiling with hydroxylamine hydrochloride and sodium acetate in alcohol

Digoxigenone, which has the same formula as XVI without the hydroxyl group in the 11-position, also, as Dr Cardwell has mentioned, has abnormal reactions with ketonic reagents It doesn't give a dioxime, but when you eliminate the 14-hydroxyl it does

CARDWELL: Prof Reichstein's ultra-violet measurements are consistent with the presence of two keto groups in sarverogenin. Our infra-red measurements in paraffin mulls disclose the presence of a six-ring keto group but do not of course determine how many such groups are present [illegible]

REICHSTEIN Yes.

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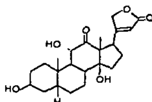
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XVI

SYNTHESIS OF 11-OXYGENATED STEROIDS FROM STEROIDAL SAPOGENINS

CARL DJERASSI and G. ROSENKRANZ

THE present paper deals chiefly with some aspects of the work done at the Syntex Research Laboratories on the conversion of steroidal sapogenins to 11-oxygenated steroids and was motivated by a desire to develop synthetic routes to cortisone and related adrenal hormones from readily available plant sources. Of these, 22a-spirost-5-en-3β-ol (diosgenin) (I) is probably one of the most important ones, and attention was directed towards the utilization of this sapogenin, which occurs (in the form of the glucoside) in many *Dioscoreæ* indigenous to Mexico and Central America.

Since 22a-spirost-5-en-3β-ol (I) possesses no substituent in ring *C* which would facilitate introduction of the requisite oxygen function at C-11, it was necessary to develop methods for the introduction of such a substituent into ring *C* unsubstituted steroids. The most feasible approach seemed through 7:9(11)-dienes, which are known to be obtainable from Δ^5-unsaturated steroids, since a point of attack is thus created in ring *C*, and the requisite dienes were therefore prepared in both the spirostan and pregnane series.

In the first instance, the conventional Wohl-Ziegler bromination with *N*-bromosuccinimide was applied to the acetate or benzoate of 22a-spirost-5-en-3β-ol (I) and after collidine dehydrobromination of the intermediate 7a-bromo derivative (II) led to the appropriate ester of 22a-spirosta-5:7-dien-3β-ol (III) (Rosenkranz, Romo and Berlin, 1951). An alternative preparation of III has been recorded recently (Ringold, Rosenkranz and Djerassi, 1952) Catalytic hydrogenation of the diene III with platinum oxide in the presence of piperidine

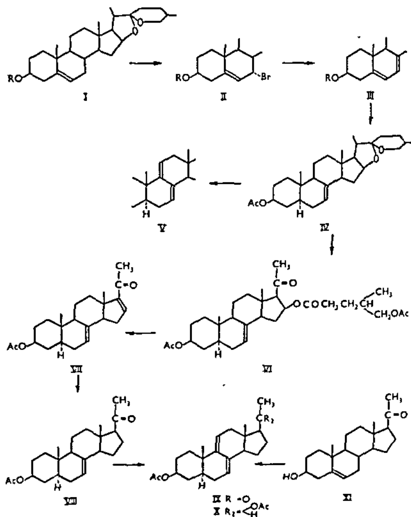
KLYNE: That might help with Prof. Shoppee's diginigenin; this has an inactive oxygen.

SHOPPEE: Yes, I think it could. Degradation products of diginigenin were examined by R. Norman Jones in the infra-red and he found no trace of an inactive ketone group.

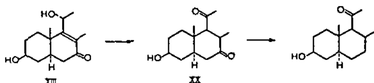
Marker (1940) via the crystalline "diosone" VI to 5α-pregna-7:16-dien-3β-ol-20-one acetate (VII), which upon catalytic hydrogenation (palladized charcoal in the presence of piperidine) to VIII, followed by a mercuric acetate dehydrogenation, produced 5α-pregna-7:9(11)-dien-3β-ol-20-one acetate (IX). This key substance (IX) could also be obtained (Djerassi, Romo and Rosenkranz, 1951) from pregn-5-en-3β-ol-20-one (XI) through the above described sequence of reactions (Wohl-Ziegler bromination, dehydrobromination, hydrogenation and mercuric acetate dehydrogenation) and thus represented an alternate route from either "diosgenin" or stigmasterol, since both steroids can be transformed on an industrial scale to XI. Furthermore, this created the essential link in the first formal total synthesis of cortisone, since both totally synthesized epiandrosterone (Cardwell, Cornforth, Duff, Holtermann and Robinson, 1951) and 3-keto-5α-etianic acid (Woodward, Sondheimer and Taub, 1951) are convertible to pregn-5-en-3β-ol-20-one (XI) (cf. Rosenkranz, Pataki and Djerassi, 1951). For the subsequent transformations to cortisone, it was considered desirable to employ 5α-pregna-7:9(11)-dien-3β:20β-diol diacetate (X) and this was accomplished by lithium aluminium hydride reduction of the 20-ketone (IX) (Romo, Rosenkranz and Djerassi, 1951).

Performic acid oxidation of the dienediol X produced 9α:11α-epoxy-5α-pregnane-3β:20β-diol-7-one diacetate (XII) which could be isomerized readily with dilute alkali to 5α-pregn-8(9)-ene-3β.11α·20β-triol-7-one (XIII) Catalytic hydrogenation to XIV, followed by removal of the 7-keto group by either the Wolff-Kishner method or desulphurization of the 7-*cyclo*ethylene mercaptal, led to 5α-pregnane-3β:11α 20β-triol (XV), further characterized by formation of a triacetate; and chromium trioxide oxidation yielded 5α-pregnane-3·11:20-trione (XVI) (Stork, Romo, Rosenkranz and Djerassi, 1951). This substance had been prepared earlier by Steiger and Reichstein (1938) from corticosterone and a direct comparison of the two specimens, kindly carried out by Prof. Reichstein, established their identity. Selective reduction of the 3-keto group could

dehydrogenated with mercuric acetate to the desired 22a-5a-spirosta-7:9(11)-dien-3β-ol acetate (V) (Rosenkranz, Romo, Batres and Djerassi, 1951). Two variations were employed to prepare the corresponding dienes in the pregnane series (Djerassi, Romo and Rosenkranz, 1951): 22a-5a-spirost-7-en-3β-ol acetate (IV) was degraded by the general procedure of



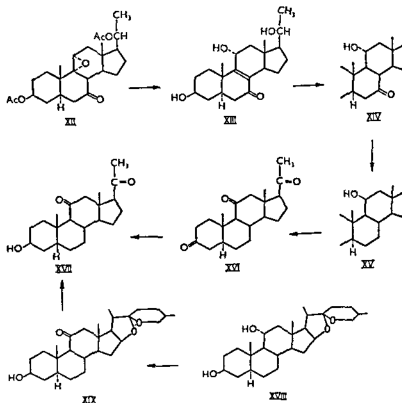
Rosenkranz, 1952). Oxidation to the 3:11-dione followed by Raney nickel hydrogenation to 22a-5a-spirostan-3β-ol-11-one (XIX) and side chain degradation (Djerassi, Batres, Romo and Rosenkranz, 1952) completed the alternate synthesis of 5a-pregnan-3β-ol-11.20-dione (XVII) from 22a-spirost-5-en-3β-ol (diosgenin) (I). The last step (XIX→XVII) has also been described by Chamberlin *et al.* (1951).



A third approach to 11-ketosteroids, which was also applied to the preparation of 5a-pregnan-3β-ol-11.20-dione (XVII), consisted of isomerization of Δ^8-7-on-11a-ols (XIII) to saturated 7 11-diones (XX) and could be accomplished in high yield by brief refluxing with potassium *tert*-butoxide in *tert*-butyl alcohol (Romo, Stork, Rosenkranz and Djerassi, 1952). The 7-keto group was removed by conversion to the 7-*cyclo*ethylene mercaptal and desulphurization with Raney nickel catalyst, and the utility of this reaction sequence was demonstrated with both 5a-pregn-8(9)-ene-3β 11a-20β-triol-7-one and 22a-5a-spirost-8(9)-ene-3β.11a-diol-7-one. A similar procedure has been outlined recently in the ergosterol series by Anderson, Budziarek, Newbold, Stevenson and Spring (1951).

5a-Pregnan-3β-ol-11.20-dione (XVII) was next transformed into 5a-pregnane-3β.17a:21-triol-11:20-dione 21-monoacetate (XXIII) (Rosenkranz, Pataki and Djerassi, 1951) by applying Kritchevsky and Gallagher's (1951) method for the introduction of the 17a-hydroxyl group (XXI) followed by bromination at C-21 (XXII) and acetolysis. The resulting monoacetate XXIII was identified as Reichstein's Compound D monoacetate by conversion to the known diacetate and direct

be accomplished with Raney nickel catalyst and thus afforded 5α-pregnan-3β-ol-11:20-dione (XVII), another key-intermediate in the synthesis of cortisone. The identical sequence of reactions was also applied to the above described 22a-5α-

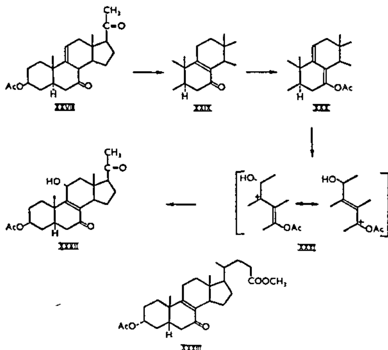


spirosta-7:9(11)-dien-3β-ol acetate (V) and proceeded through intermediates analogous to XII,* XIII and XIV to 22a-5α-spirostan-3β:11α-diol (XVIII) (Djerassi, Batres, Velasco and

steiner and Pfiffner, 1935; Compound A of Reichstein, 1936; Compound D of Mason, Myers and Kendall, 1936).

In order to complete the synthesis of cortisone, it was necessary to introduce the essential 4:5-double bond and this was accomplished by applying the general method of Rosenkranz, Djerassi and co-workers (1950) for the conversion of 3-keto-5α-steroids to Δ^4-3-ketones to the specific case at hand (Rosenkranz, Djerassi, Yashin and Pataki, 1951). Dibromination of XXIV gave the 2:4-dibromo derivative XXVI which, without isolation, was refluxed with sodium iodide in acetone solution and then deiodinated with sodium bisulphite to afford cortisone acetate (XXVII).

In the above described syntheses of 11-oxygenated steroids from the ring *C*-unsubstituted starting material 22*a*-spirost-5-en-3β-ol (I), the key step is the performic acid oxidation of an



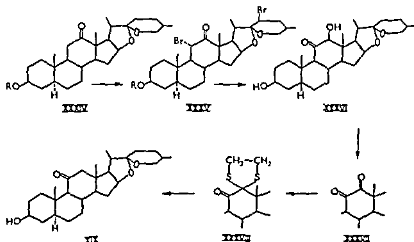
comparison with an authentic sample obtained from beef adrenal glands (Euw and Reichstein, 1942). This represents the first partial synthesis of Reichstein's Compound D and was reported independently also by Chemerda, Chamberlin,

Wilson and Tishler (1951). Oxidation of the 21-monoacetate (XXIII) with *N*-bromoacetamide in pyridine solution yielded 5α-pregnane-3:11:20-trione-17α:21-diol 21-acetate (XXIV) ("dihydro*allo*cortisone acetate") and subsequent lithium aluminium hydride reduction (Djerassi, Rosenkranz, Pataki and Kaufmann, 1952) produced 5α-pregnane-3β.11β:17α:20β.21-pentol (XXV), a substance which has previously been available only from adrenal glands (Compound A of Winter-

The class of Ring *C* oxygenated sapogenins, occurring primarily in *Agave* and *Yucca* species represents another potentially very important source of raw material for cortisone syntheses and this will be exemplified with the most abundant member of this series, 22*a*-5*a*-spirostan-3*β*-ol-12-one (hecogenin) (XXXIV). Since the corresponding 11-keto analogue (XIX) (*vide supra*) has already been transformed to cortisone (XXVII), it was only necessary to effect the shift of the 12-keto group to the 11-position in order to complete the synthetic sequence from "hecogenin" (XXXIV) to cortisone. Of the various methods developed in the bile acid series for accomplishing this change, only that of Gallagher (Gallagher *et al*, 1946, Borgstrom and Gallagher, 1949) appeared suitable. It proved successful in its initial stages (Djerassi, Martinez and Rosenkranz, 1951*a*) in that it was found possible to dibrominate 22*a*-5*a*-spirostan-3*β*-ol-12-one acetate (XXXIV) to the 11·23-dibromo derivative XXXV and to hydrolyse the latter (followed by debromination at C-23) with the formation of 22*a*-5*a*-spirostan-3*β*. 12*β*-diol-11-one (XXXVI). While no difficulty was encountered in selectively acylating the C-3 hydroxyl group, subsequent treatment with phosphorus tribromide in an attempt to replace the C-12 hydroxyl group by bromine resulted in side reactions with the spiroketal side chain. The following alternative procedure was developed (Djerassi, Ringold and Rosenkranz, 1951) which has also proved applicable to the bile acids: oxidation of the ketol XXXVI with bismuth oxide (Rigby, 1951) led to 22*a*-5*a*-spirostan-3*β*-ol-11:12-dione (XXXVII), which could be condensed with ethanedithiol in the presence of hydrogen chloride to yield the 12-*cyclo*ethylene mercaptal (XXXVIII). Desulphurization with Raney nickel catalyst in the usual manner gave the desired 22*a*-5*a*-spirostan-3*β*-ol-11-one (XIX) which has already been converted to cortisone.

The above described methods for the introduction of an oxygen function into ring *C* unsubstituted steroids possess the attractive feature of proceeding through 11*a*-hydroxy intermediates and we have carried out, therefore, the chemical

allo(5α)-$\Delta^{7,9(11)}$-diene to the corresponding 9α:11α-epoxy-7-ketone (e.g. XII). This reaction is applicable only to 5α (*allo*) steroids, since the corresponding 5β-(normal) $\Delta^{7,9(11)}$-dienes give exclusively the $\Delta^{8(9)}$-7-ketone (Fieser, Babcock, Herz, Huang and Schneider, 1951). In fact, such $\Delta^{8(9)}$-7-ketones (XXIX) are also obtainable from the by-products (e.g. XXVIII) in the performic acid oxidation in the 5α(*allo*) series or the dichromate oxidation in both the 5α and 5β series (Fieser, Herz and Huang, 1951), as well as by acidic rearrangement of Δ^{7}-9α:11α- epoxides (Heusser *et al.*, 1952; Schoenewaldt *et al.*, 1952) and it has been possible to use such compounds for the synthesis of 11-oxygenated steroids (Djerassi, Mancera, Stork and Rosenkranz, 1951). Alkaline isomerization of the unconjugated ketone XXVIII leads to the conjugated isomer XXIX which is transformed to the enol acetate XXX and then treated with perbenzoic or monoperphthalic acid to yield directly the 3-acylated $\Delta^{8(9)}$-7-one-11α-ol (XXXII); species such as XXXI are believed to be responsible for this smooth transformation, which has been found to be equally applicable (Djerassi, Mancera, Velasco, Stork and Rosenkranz, 1952) to 5β-derivatives such as Fieser's (1951) methyl-3α-acetoxy-7-ketochol-8(9)-enate (XXXIII).



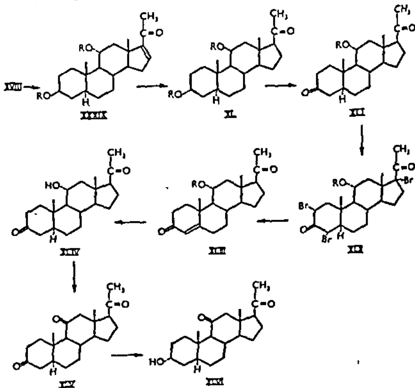
one (XL) into the corresponding 11α-ol-3:20-dione (XLI) and then to introduce the 4·5-double bond by our sodium iodide procedure (Rosenkranz, Djerassi *et al.*, 1950) on the 2:4:17-tribromo derivative (XLII) yielding 11α-hydroxyprogesterone (XLIII) (Mancera, Romo, Sondheimer, Rosenkranz and Djerassi, 1952). Similar procedures were used, starting with both XXXIX and XL, for the synthesis of the corresponding analogues of corticosterone and 17α-hydroxycorticosterone (Kendall's compound F) (Romo, Zaffaroni, Hendrichs, Rosenkranz, Djerassi and Sondheimer, 1952).

Since the completion of this work there appeared a communication by Peterson and Murray (1952) in which was reported the microbiological oxidation of progesterone with the mould *Rhizopus arrhizus* in 10 per cent yield to a substance believed to be 11α-hydroxy-progesterone (XLIII) The physical constants of the microbiological oxidation product proved to be in excellent agreement with those found in our laboratory for the chemically synthesized substance. Employing an as yet unidentified strain of *Rhizopus* isolated from a Mexican soil sample, it has been found possible (Māncera, Zaffaroni, Rubin, Sondheimer, Rosenkranz and Djerassi, 1952) to raise the yield in the microbiological oxidation to *ca.* 45 per cent. Furthermore, in striking contrast to the catalytic reduction of 11-keto and 11β-hydroxy Δ^4-3-ketosteroids which produces chiefly the 5α(*allo*) dihydro derivative (cf. Pataki, Rosenkranz and Djerassi, 1952), it was observed that similar hydrogenation of 11α-hydroxyprogesterone (XLIII) produced the 5β(normal) analogue, pregnane-3·20-dion-11α-ol (XLIV), which upon chromium trioxide oxidation to pregnane-3:11·20:trione (XLV) followed by sodium borohydride reduction furnished in good yield pregnane-11:20-dion-3α-ol (XLVI). The latter has already been converted to cortisone (Kritchevsky, Garmaise and Gallagher, 1952) and it appears, therefore, that both in terms of number of steps (ten from progesterone or fourteen from diosgenin) and overall yield the combined microbiological-chemical sequence represents the as yet best described route to cortisone.

synthesis of a number of previously unknown 11α-epimers of certain 11β-hydroxy cortical hormones. A brief outline of certain aspects of this work follows:—

The key starting material was 22a-5α-spirostan-3β.11α-diol (XVIII) Djerassi, Batres, Velasco and Rosenkranz, 1952), which could be degraded in the usual manner to 5α-pregn-16-ene-3β:11α-diol-20-one (XXXIX) and then hydrogenated to 5α-pregnane-3β.11α-diol-20-one (XL) (Djerassi, Batres, Romo and Rosenkranz, 1952). It was then found that (*a*) a 3β:11α-diol can be selectively oxidized (*N*-bromosuccinimide or Oppenauer oxidation) to the 3-one-11α-ol and (*b*) that a 3β:11α-diol diacetate can be selectively saponified at C-3.

Taking advantage of these observations, it proved a relatively simple matter to convert 5α-pregnane-3β:11α-diol-20-



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DISCUSSION

In conclusion, it may be pointed out that there are available now three main routes to cortisone from potentially inexhaustible plant sources—the use of diosgenin or hecogenin in completely chemical syntheses or a combined microbiological-chemical approach employing a diosgenin transformation product.

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Reichstein's Compound D to cortisone, namely oxidation to the 3-keto group, dibromination, treatment with sodium iodide and de-iodination. The resulting 11α-F diacetate has been prepared independently by Prof. Reichstein from sarmentogenin, and, as he said, there may be other syntheses as well.

H HIRSCHMANN. How does the "11α-corticosterone" compare with the

Reichstein (*Helv. chim. Acta,*

I believe in the light of present

this preparation had some

sical constants of the two

DJERASSI· I am afraid I do not remember them. I think Prof. Reichstein is in a better position to tell you.

REICHSTEIN One of the reasons why we made this compound was

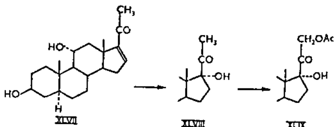
diacetate is very difficult to crystallize with small quantities. Perhaps you have made the same observations It was synthesized five years ago or earlier by Gallagher (*Recent Progr Hormone Res* , 1947, 1, 95), but he didn't publish details because his acetate did not crystallize. Ours took about three months to form the first crystals; but once it has been ob-

ketol side chain Now as long as the other hydroxyl group at C-17 is not present, as in 11-epicorticosterone diacetate, we found it possible to saponify the 11α-acetoxy group by several hours of boiling with hydrochloric acid in methanol; but in the 11-epi-17-hydroxycorticosterone diacetate, the side chain is sensitive to both strong alkali and strong acids. We haven't even tried to saponify it by purely chemical means. We wanted to do it with enzymes and though we now have enzymes which may be appropriate to do it, it hasn't been done yet, at least by us I would be interested to know whether you have succeeded in saponifying the 11-acetoxy group without destroying the side chain.

DJERASSI· We do not yet have the crystalline compound. We plan to use the hydrolysed material directly for biological testing Perhaps Dr. Sondheimer, who has just come from Mexico, can answer this.

manufacture has been very largely in ...

easily obtained from the juice, and it does seem that it can be made a readily available and cheap raw material. There is a pilot plant at Nairobi which is producing about 1 kg of hecogenin crude concentrates a week. I may say that we await with interest further results from the Djerassi-Rosenkranz school in planning what to do with the hecogenin. We have done a certain amount—it is Dr. Cornforth's work—but I don't think it is yet ready for publication.



DJERASSI: I wish to mention a point which I forgot to bring up in my talk. I s...
mones; these ...
progesterone, ...
that Prof Reichstein prepared from sarmentogenin) and the corticosterone derivative. Some of the compounds were prepared, not only for biological evaluation, but also as intermediates for Dr. Zaffaroni, who has been doing a number of enzymic and other transformations on such derivatives and has obtained some exceedingly interesting results. The most important part is described in a paper which has ...

with chemists, but the microbiological work will give us certain starting materials which we have never had before; it will make available certain very valuable analogues of some of the important adrenal hormones. For the time being the presently described method through progesterone gave by far the best yields of cortisone. That's why I think that the ultimate synthesis of cortisone will be a combined microbiological and chemical sequence, but not necessarily the one that I showed.

I ought to add that there was one other person who played a very

CORTI.

acid curves, which are quite characteristic of the various corticosteroids, are completely identical for 11α- and the 11β-hydroxysteroids. He found this in about 6 or 7 pairs of epimers. Hence the sulphuric acid curves, coupled with the behaviour on paper chromatography, ought to show whether even the crude saponification product used for that biological work was the expected product. Paper chromatography has shown that the 11α-hydroxy compound is more polar than the 11β-hydroxy, which again would be expected—not polar in the Bartonian sense, but polar on paper.

Furthermore, Dr. Zaffaroni has prepared some of the free 11α-F by a biochemical procedure which is included in our communication to *Chemistry and Industry* (1952, p. 783). He observed that oxidation of 11α-hydroxyprogesterone with adrenal brei produced both the free 11α-corticosterone and 11α-Compound F.

SONDHEIMER: That is quite correct, but there is also another way to prepare the 11-epimer of Compound F, not acetylated at C-11. We have made the free 11-hydroxy-"dihydro-*allo*" compound (5α-pregnane-$11\alpha{:}17\alpha{:}21$-triol-3:20-dione 21-monoacetate) by a route similar to that described by Dr. Djerassi for the 11:21-diacetate. Double bond introduction into this substance will give *11-epi-17α-hydroxycorticosterone* 21-acetate, where no problem of hydrolysis exists (cf. *J. Amer. chem. Soc*., 1953, 75, 1277).

REICHSTEIN: You said that you always take progesterone for the starting material for your microbiological introduction of the hydroxy group in the 11α-position and yet this is not convenient afterwards. It seems a little queer, and I think the reason is that you probably get the best results with progesterone itself.

DJERASSI: You mean the microbiological oxidation?

REICHSTEIN: Yes. Is that true, or was it only tried with those compounds? For instance, you said you get something with pregnenolone, but is the yield really much lower?

DJERASSI: Again I am only speaking for others, because obviously this is not work that the chemical group has done, but rather the biochemical group under Dr. Zaffaroni and Dr. Rubin. They have oxidized nearly all the readily available steroids that we have, from diosgenin down, and in order not to waste much time they have analysed them by micro methods on paper. This indicated that just about every one was oxidized to a certain extent. The yield with this particular micro-organism was the highest in the case of progesterone. We have done it with other compounds, especially Compound S. Compound S produces the 11α-hydroxy analogue, which can be used for the synthesis of cortisone only if you would want to oxidize afterwards.

It would be unfair for me to talk about this wealth of material, to

with chemists, but the microbiological work will give us certain starting materials which we have never had before; it will make available certain very valuable analogues of some of the important adrenal hormones. For the time being the presently described method through progesterone gave by far the best yields of cortisone. That's why I think that the ultimate synthesis of cortisone will be a combined microbiological and chemical sequence, but not necessarily the one that I showed.

I ought to add that there was one other person who played a [illegible]

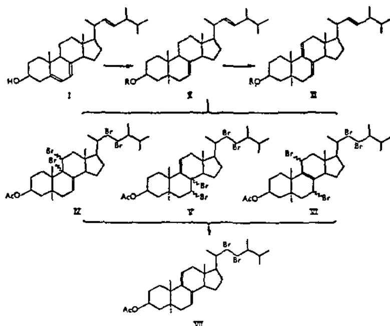
11-OXYGENATED STEROIDS FROM ERGOSTERYL-D ACETATE 22:23-DIBROMIDE

F. S. SPRING, G. T. NEWBOLD, R. C. ANDERSON, R. BUDZIAREK, F. JOHNSON, J. A. McEWAN, D MACLEAN and R. STEVENSON

At an early stage in the investigation of syntheses of 11-oxygenated steroids starting from ergosterol (ergosta-5:7:22-trien-3β-ol) (I), the availability of ergosterol-D (III; R=H) became a matter of considerable concern. The most practicable route previously described appeared to consist in partial hydrogenation of ergosteryl esters in a neutral solvent (Heilbron and Sexton, 1929; Wieland and Benend, 1943; Barton and Cox, 1948) to the corresponding 5-dihydroergosteryl ester (II) followed by oxidation of the latter with mercuric acetate (Windaus and Auhagen, 1929; Heilbron, Johnstone and Spring, 1929). The first stage of this process was considerably improved: using benzene as solvent and Raney nickel as catalyst, nearly quantitative yields of 5-dihydroergosteryl acetate (II; R=Ac) are obtained from ergosteryl acetate (Anderson, Budziarek, Newbold, Stevenson and Spring, 1951). Similar improvements have been reported by Heusser, Eichenberger, Kurath, Dällenbach and Jeger (1951) using ether as solvent and Rupe nickel. More recently Laubach and Brunings (1952) have effected the same reaction in similar yield using Raney nickel and dioxan as solvent.

The oxidation of 5-dihydroergosteryl acetate to ergosteryl-D acetate (III, R=Ac) using mercuric acetate is an inefficient process; although a crude reaction product is isolable in reasonable yield, the purification of this product is attended by serious loss, the yield of ergosteryl-D acetate with $[\alpha]_D \nless 28°$ being of the order of 30 per cent.

In a search for a more efficient method for the preparation of ergosteryl-D acetate, a study was made of the action of bromine on 5-dihydroergosteryl acetate, since Eck and Hollingsworth (1942) had shown that treatment of cholest-7-ene with bromine at −75° gives cholesta-7:9(11)-diene. Treatment



of 5-dihydroergosteryl acetate in ether at—60° with bromine in acetic acid gives in 50 per cent yield a crystalline tetrabromoergostenyl acetate. This tetrabromide is moderately stable in the solid state but solutions in chloroform suffer profound decomposition after a short time at room temperature. When treated with sodium iodide, the tetrabromide is quantitatively converted into the stable and beautifully crystalline ergosteryl-D acetate 22:23-dibromide [22 23-dibromoergosta-7·9(11)-dien-3β-yl acetate] (VII), the structure of which was established by debromination with zinc into

ergosteryl-D acetate and by its ultra-violet absorption spectrum, which in general character is identical with that of ergosteryl-D acetate.

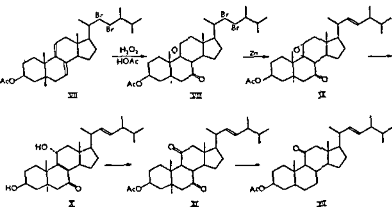
The structure of the tetrabromoergostenyl acetate has not been established beyond reasonable doubt. Its ready conversion into ergosteryl-D acetate 22:23-dibromide by treatment with sodium iodide and its formation from ergosteryl-D acetate by the action of bromine, limit the possibilities to 9:11:22:23-tetrabromoergost-7-en-3β-yl acetate (IV), 7:8:22:23-tetrabromoergost-9(11)-en-3β-yl acetate (V) or 7:11:22:23-tetrabromoergost-8-en-3β-yl acetate (VI). Catalytic reduction of tetrabromoergostenyl acetate in dioxan, using a platinum catalyst, gives ergosteryl-D acetate 22:23-dibromide.

The bromine method is an attractive route to ergosteryl-D acetate since the conversion of tetrabromoergostenyl acetate into ergosteryl-D acetate 22:23-dibromide is quantitative, as is the conversion of the latter into ergosteryl-D acetate. Ergosteryl-D acetate obtained by this method is of high purity. The bromine method can be used for the preparation of ergosteryl-D acetate without isolation of either the tetrabromo- or dibromo-intermediates. Treatment of 5-dihydroergosteryl acetate with bromine followed by debromination of the mixture with zinc gives ergosteryl-D acetate in 70 per cent yield.

The oxidation of a Δ^7-steroid with bromine appears to be a general reaction. When applied to α-spinasteryl acetate, obtainable from either Lucerne grass (alfalfa) or from stigmasterol using the procedure described by Fieser, Fieser and Chakravarti (1949), an unstable tetrabromostigmastenyl acetate is obtained which with sodium iodide yields 22:23-dibromostigmasta-7:9(11)-dien-3β-yl acetate, debromination of which with zinc dust gives stigmasta-7:9(11):22-trien-3β-yl acetate, identical with a specimen obtained by the action of mercuric acetate on α-spinasteryl acetate. The formation of this triene is yet another proof of the correctness of the

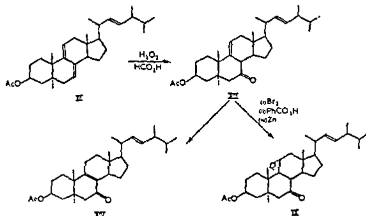
acetate with chlorine
corresponding reaction

with bromine. A mixture of two tetrachloroergostenyl acetates results. Of these, tetrachloroergostenyl acetate I is converted into 22:23-dichloroergosta-7:9(11)-dien-3β-yl acetate by treatment with sodium iodide, and is the analogue of tetrabromoergostenyl and tetrabromostigmastenyl acetates. The other is a secondary product and is related not to ergosterol-D, but to ergosterol-B_3.



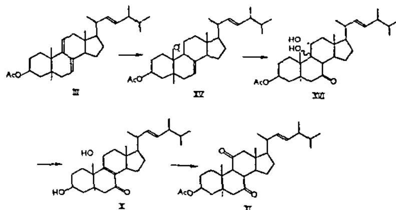
The preparation of ergosteryl-D acetate 22:23-dibromide makes possible the oxidation of the nuclear ethylenic linkages in ergosteryl-D acetate without complications arising from the presence of the side chain ethylenic linkage Using hydrogen peroxide in acetic acid, oxidation of the dibromide gives 3β-acetoxy-22.23-dibromo-9α:11α-epoxyergostan-7-one (VIII) debrominated by means of zinc to 3β-acetoxy-9α:11α-epoxyergost-22-en-7-one (IX) which can also be obtained in poor yield from ergosteryl-D acetate by oxidation with performic acid. The structure of (VIII) is established by the general correspondence in properties with those of analogously constituted compounds obtained by Djerassi *et al.* (1951) by performic oxidation of 7:9(11)-dienic steroids. It shows no selective absorption of high intensity above 2,200A, but it shows carbonyl absorption at 1,710 cm.$^{-1}$, and mild alkaline hydrolysis

of (IX) gives 3β:11α-dihydroxyergosta-8:22-dien-7-one (X). When treated with 15 per cent alcoholic potassium hydroxide, it gives, after acetylation, a mixture from which 7:11-diketo-ergost-22-en-3β-yl acetate (XI) was isolated. The latter is converted into 11-ketoergost-22-en-3β-yl acetate (XII) as described by Chamberlin *et al.* (1951). The oxidation of ergosteryl-D acetate with one mole of performic acid gives as first isolable product 3β-acetoxyergosta-9(11):22-dien-7-one (XIII), the structure of which follows from its lack of high intensity absorption above 2,200Å, from its carbonyl absorption in the infra-red (1,715 cm^{-1}.) and from the ease with which it is isomerized to 3β-acetoxyergosta-8:22-dien-7-one (XIV). Further oxidation of 3β-acetoxyergosta-9(11):22-dien-7-one, after protection of the side chain ethylenic linkage with bromine, with perbenzoic acid followed by debromination gives once again 3β-acetoxy-9α:11α-epoxyergost-22-en-7-one (IX).



Two distinct compounds have now been ascribed the same structure (XIII); they differ in orientation at C_8. Each has been characterized by facile conversion into 3β-acetoxy-ergosta-8:22-dien-7-one and by characteristic infra-red carbonyl absorption.

A related oxidation product is worthy of mention. Treatment of ergosteryl-D acetate with perbenzoic acid gives the 9α:11α-epoxide (XV) (Chamberlin *et al.*, 1951; Heusser *et al.*, 1951), which when successively treated with bromine (1 mole) and excess perbenzoic acid followed by debromination with zinc yields 3β-acetoxy-9ξ:11α-dihydroxyergost-22-en-7-one (XVI), characterized by acetylation and by its ready con-



version into (X) by treatment with alkali, which as shown previously can be converted into 11-ketoergost-22-en-3β-yl acetate (XII) through the intermediate 7:11-diketoergost-22-en-3β-yl acetate (XI).

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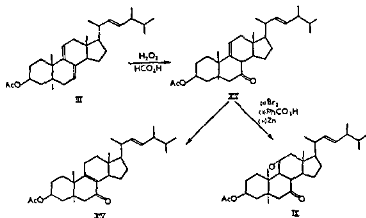
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of (IX) gives 3β:11α-dihydroxyergosta-8:22-dien-7-one (X). When treated with 15 per cent alcoholic potassium hydroxide, it gives, after acetylation, a mixture from which 7:11-diketoergost-22-en-3β-yl acetate (XI) was isolated. The latter is converted into 11-ketoergost-22-en-3β-yl acetate (XII) as described by Chamberlin *et al.* (1951). The oxidation of ergosteryl-D acetate with one mole of performic acid gives as first isolable product 3β-acetoxyergosta-9(11):22-dien-7-one (XIII), the structure of which follows from its lack of high intensity absorption above 2,200Å, from its carbonyl absorption in the infra-red (1,715 cm^{-1}.) and from the ease with which it is isomerized to 3β-acetoxyergosta-8:22-dien-7-one (XIV) Further oxidation of 3β-acetoxyergosta-9(11) 22-dien-7-one, after protection of the side chain ethylenic linkage with bromine, with perbenzoic acid followed by debromination gives once again 3β-acetoxy-9α:11α-epoxyergost-22-en-7-one (IX).



Two distinct compounds have now been ascribed the same structure (XIII), they differ in orientation at C_8. Each has been characterized by facile conversion into 3β-acetoxyergosta-8:22-dien-7-one and by characteristic infra-red carbonyl absorption.

reflux for a while to form the hydrazone, then distil out enough water
to raise the temperature to 200°, and then reflux 8 hours more and

the autoclave is not always necessary.

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DISCUSSION

[illegible]

DJERASSI· We never tried 15 per cent. With 5 per cent it does not go, because those are really the conditions under which the epoxyketone rearranges to the unsaturated ketol. Acidic reagents, on the other hand, result in dehydration. I think this is paralleled by Ehrenstein's observation on the isomerization of epimeric 6-hydroxy-Δ^{4}-3-ketones the 6α does not go readily while the 6β is isomerized very readily. We then [illegible]

SPRING· No, not yet.

ANNER As far as I know, the yield of the performic acid oxidation of 7:9(11)-dienes hasn't been given in the literature. Could you tell me how much you obtained?

SPRING: We haven't processed the various stages with a view to maximum yield It's of the order of 40 or 50 per cent.

FIESER I might mention one item of general interest from the experimental point of view A number of speakers here have mentioned the use of the Huang-Minlon procedure for Wolff-Kishner reduction (Huang-Minlon, *J. Amer. chem. Soc* , 1946, 68, 2487; 1949, 71, 3301) [illegible]

reduction of the keto group of the aroylpropionic acid. We tried this by the Clemmensen method and the best yield was 54 per cent. Two different very able workers tried the thing, but couldn't get beyond that result. We wanted to make this compound in huge amounts, for clinical trial and so on, and so we didn't like this limitation of 54 per

"β-oxide," m.p. 115·5°–117·5°, was reported to be identical with the oxide obtained by the peracid oxidation of the parent sterol.* The higher-melting "oxide", m.p. 146–146·5°, was not completely characterized but was shown to be inert to chromic acid and to perbenzoic acid. Neither "oxide" could be cleaved by catalytic reduction or by treatment with the common mineral acids.

In the course of the present investigation, we also have obtained two compounds from the same oxidation. The higher melting compound, m.p. 147°, was identified as methyl 3α-acetoxy-12-ketochol-9(11)-enate (III) rather than an epimeric oxide. Its structure was elucidated by ultra-violet absorption analysis λ (max. 240 mμ; log ε 4·04) and by direct comparison with an authentic sample. The lower-melting compound (II), obtained as the major product in our investigation, showed a pronounced depression of melting point upon admixture with an authentic sample of methyl 3α-acetoxy-9α:11α-epoxy-cholanate obtained by peracid oxidation of the parent olefin; and its melting point and specific rotation were at variance with those previously reported for the α-oxide (Seebeck and Reichstein, 1943; Fieser and Rajagopalan, 1951, Berner and Reichstein, 1946).

Because of the impossibility of obtaining *prima facie* evidence of the existence of the oxide group as such, the structure of this oxide† must be inferred from its elementary analysis, which shows an extra oxygen atom in the molecule, its infra-red absorption spectrum, which shows no recognizable oxygen-function absorption not previously present in the starting material; from its dissimilarity to the known α-oxide; and finally, from its conversion to the known methyl 3α-acetoxy-

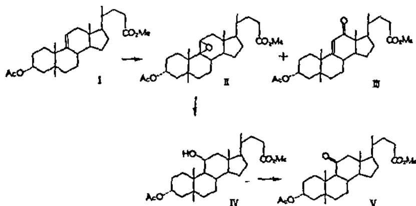
*At the time this paper appeared [illegible]

†It is of interest to note that the preparation of this oxide represents the first transformation of a steroid to a derivative possessing a substituent at C9 which is unequivocally in the β orientation

INTRODUCTION OF THE 11-KETO FUNCTION IN THE STEROIDS

J. M CONSTANTIN and L. H. SARETT

BECAUSE of the increasing interest in both total and partial synthesis of the adrenocortical hormones, the conversion of $\Delta^{9(11)}$ steroids to 11-oxygenated derivatives has recently received considerable attention (Chamberlin *et al.*, 1951; Stork *et al.*, 1951; Heusser *et al.*, 1951; Fieser and Heymann, 1951; and preceding papers). With this impetus, an investigation initiated by one of us (L.H.S.) in 1942, involving the permanganate oxidation (cf. Ehrenstein and Decker, 1940) of a $\Delta^{9(11)}$ steroid in acidic solution, has recently been extended and brought to fruition with the synthesis of methyl 3α-acetoxy-11-ketocholanate (V).



During the intervening years, one publication appeared (Hicks, Berg and Wallis, 1946) reporting the formation of two oxides upon oxidation of methyl 3α-acetoxychol-9(11)-enate with potassium permanganate in acetic acid. The so-called

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DISCUSSION

BARTON. I should like to ask Dr. Sarett if he has opened the β-oxide with hydrogen halide to obtain the 11β-hydroxy-9α-halogen compound.

SARETT. No That hasn't been done

BARTON: The oxide ring has only been opened by catalytic hydrogenation?

SARETT. Yes. If one could open to the halohydrin there, it would be most interesting.,

BARTON: One would predict, I think, the opposite opening for the β-oxide to what one would expect with the α-oxide.

SARETT: Yes, I think that's right.

DJERASSI. But the α-oxide wouldn't give it to you either.

BARTON No, the α-oxide's inert—but *if* it were reactive it would open the other way.

DJERASSI Doesn't the 9α:11α-oxide give you a diene under strong enough conditions?

FIESER: Yes, with methanol and hydrochloric acid.

DJERASSI: Or with strong HBr.

ROBINSON: Have these substances been subsequently treated to

bile
be

REICHSTEIN I would like to ask Dr. Sarett a question about the α-oxide—how strongly he treated with $LiAlH_4$. Did he use lithium aluminium hydride in ether or has he treated it under more vigorous conditions too?

DJERASSI We have attempted this reduction in tetrahydrofuran, refluxing overnight, and still there was no reaction This was in the sapogenin series, particularly the example that Dr. Sarett mentioned.

FIESER. What about HBr treatment?

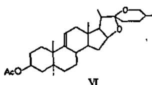
DJERASSI· We were so discouraged by your results in the 5β series that the only experiment we performed was reduction with lithium aluminium hydride. We did not try HBr. Of course the trouble with

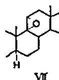
that I talked about yesterday.

DJERASSI· In some other cases where we had no oxide and treated a sapogenin structure with HBr, we got introduction of bromine into

11β-hydroxycholanate (IV) (Wintersteiner and Moore, 1946; Turner *et al.*, 1946).

In contrast to the inertness of the 9α:11α-oxides (Hicks, Berg and Wallis, 1946; Berner and Reichstein, 1946), the β-oxide readily underwent hydrogenolysis with platinum in acidic solution. Finally, oxidation of this product with chromium trioxide in acetic acid gave methyl 3α-acetoxy-11 keto-cholanate (V) in good yield.





To test the scope of this oxidation procedure, an *A/B trans* steroid, specifically, $\Delta^{9(11)}$- tigogenin acetate (VI), was oxidized under similar conditions, and gave in excellent yield the same oxide (VII) as that obtained by peracid oxidation. The latter oxide has been assigned the 9α:11α configuration by Djerassi *et al.* (1951), and has been reported by these authors to be inert to a variety of reagents, further substantiating the characteristic inertness of the isolated 9α·11α oxides.

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FIESER, L F., and RAJAGOPALAN, S (1951). *J Amer. chem. Soc.*, **73**, 118
R.,

DJERASSI What about $LiAlH_4$ and the β-oxide—did you get anything with that?

SARETT: We only got the β-oxide at first in the bile acid series. We knew we'd be having a primary hydroxyl group right on the end, so we didn't try it.

oxides. I suppose it's related to the inert ketone Does the ketone group show the ordinary infra-red spectrum?

SARETT Yes, at 5 84 μ It shifts around a little bit, and of course, it's at a different frequency from the cyclohexanone, or Ring *A* ketone, but not by much.

This "internal" reaction, so to speak, described by Prof. Fieser and

one in which the thing's purely intramolecular.

the side chain, presumably in the 16 and 26 positions. But this would

compound melting at about 245° which seemed by infra-red to be definitely different from the 263° oxide, and yet didn't contain a hydroxyl group. We couldn't do anything with the compound, but it did change somehow with $LiAlH_4$. Would that be the side chain?

DJERASSI. No

SARETT· We gave it a hard going-over

ROBINSON: The formula on the board is that of an acetoxy compound. Are we talking about acetoxy compounds all the time? Because, if we are, the $LiAlH_4$ will reduce that in any case

DJERASSI Yes, but he re-acetylated it afterwards.

ROBINSON If you acetylate after the reduction, that would also acetylate the hydroxyl group that you're trying to find

DJERASSI: The only one we tried was just the 9.11 epoxide I've forgotten whether this was on the acetate or the free compound, but that would be immaterial, because the product was identical with the starting material.

ROBINSON: Yes, I'm only trying to clear up the point that Dr. Sarett mentioned concerning the substance which showed no infra-red band due to a hydroxyl group.

SARETT. That's correct—it was back-acetylated. Then your point about acetylating a new hydroxyl group comes up. The problem isn't as simple as I indicated at first We went around with oxidations and partial oxidations and the infra-red spectra of resulting ketones and so on, and I think we're able to prove that we never had an additional hydroxyl group produced in the molecule.

CARDWELL: Was your $LiAlH_4$ quite free from $AlCl_3$?

SARETT. I don't know. Is that important?

DJERASSI. Yes Fontaine (*J. Amer. chem Soc.*, 1951, 73, 5917) found that if a sapogenin is reduced with $LiAlH_4$ in the presence of HCl, the side chain is opened to the dihydrosapogenin This would not affect your oxide, but the dihydrosapogenin would now have a free hydroxyl group at the 26 position.

ROBINSON· I think the point that Dr. Cardwell just raised is interesting It arises from some work at Oxford of Mr Brown, who is now at Cambridge, which has not yet been published (cf *J chem Soc*, 1952, p. 2756—published after the conference) This showed that $LiAlH_4$ made in the laboratory from LiH and aluminium chloride behaved quite differently from purchased $LiAlH_4$ in the cholest-4-en-3-one series (Dauben and Eastham, 1951, *J. Amer. chem. Soc.*, 73, 3260) He obtained some very peculiar results, involving the stringing of an oxygen atom across the nucleus somewhere. The only point that I

very weak, failures to detect them have been reported with several Δ^5-unsaturated steroids (Jones *et al.*, 1948; Josien, Fuson and Cary, 1951) In contrast, the bending vibrations of olefinic hydrogens give rise to much stronger bands. Their frequency is profoundly affected by the number of hydrogen atoms attached to the double bond. If only a single olefinic hydrogen is present an absorption band is observed between 800 and 840 cm $^{-1}$ (Thompson and Torkington, 1945; Barnard *et al.*, 1950). Maxima associated with the motions of saturated linkages can also occur in this region but these are generally considered to be much weaker (Rasmussen, 1948). When this study was started no systematic investigation of steroidal olefines in this region had been recorded, but after our data had been collected, Bladon, Fabian, Henbest, Koch and Wood (1951*a*) reported a thorough analysis of band frequencies and intensities of relatively simple trisubstituted steroidal olefines, their reduction products and their isomers with tetrasubstituted ethylene groups Although no overlap of band intensities was noted they cautioned that bands of intermediate strength might be diagnostically unreliable. However, they considered extreme weakness of a band in this region (790–850 cm.$^{-1}$) a dependable criterion for the absence and strong bands as a fairly safe indication for the presence of the trisubstituted olefinic linkage in the steroid molecule.

In view of the importance of the height of absorption maxima only curves obtained on solutions will be presented, as the effective concentration of solids in the light path is hard to assess. The transmission spectrum of the solvent, carbon disulphide (Fig. 1) shows a gradual rise with increasing frequency which is due to increased radiation intensity of the source, followed by a steep fall caused by an absorption maximum of the solvent at 855 cm. $^{-1}$ (American Petroleum Institute catalogue). This peak can easily obscure weak maxima of the solute especially if rather dilute (1 per cent) solutions are examined as was done here. However, this choice of experimental conditions was urged upon us by the low solubility and the scarcity of many compounds investigated.

THE CHARACTERIZATION OF TRISUBSTITUTED STEROIDAL OLEFINES BY INFRA-RED SPECTROSCOPY

H. HIRSCHMANN

THE identification of a new steroidal metabolite, if it has to be carried out with a very small amount of material, frequently requires a fairly detailed picture of the probable structure before a successful verification by chemical means can be undertaken. A great deal of this preliminary information is usually obtained by measurements of physical properties. As the absorption of radiant energy and the rotatory power of unsaturated steroids are often affected by the location of the double bond and the substituents in its vicinity a fairly reliable diagnosis of the position of the double bond can often be made if the substituents in its environs and their effect on absorption or rotation are known. In the absence of such information only a consistent answer in a variety of independent tests can give comparable assurance. It is, therefore, with a view to supplementing rather than to replacing existing procedures that our findings in the 12μ region of infra-red radiation are being reported.

Quite generally, measurements in three regions of infra-red radiation have furnished tools for diagnosing the presence of partially substituted double bonds and the extent of their aliphatic substitution. They are associated with the stretching motions of the double bond and with the stretching and out-of-plane bending motions of the hydrogen bonds attached to it. Absorption bands related to these stretching vibrations of unsaturated steroids have been investigated chiefly by Jones *et al.* (1948, 1950) and by Bladon *et al.* (1951*a*). The bands appear to be quite characteristic of the position of the double bond but as the maxima or inflexions are generally

very weak, failures to detect them have been reported with several Δ^5-unsaturated steroids (Jones *et al.*, 1948; Josien, Fuson and Cary, 1951). In contrast, the bending vibrations of olefinic hydrogens give rise to much stronger bands. Their frequency is profoundly affected by the number of hydrogen atoms attached to the double bond If only a single olefinic hydrogen is present an absorption band is observed between 800 and 840 cm.[1] (Thompson and Torkington, 1945; Barnard *et al.*, 1950). Maxima associated with the motions of saturated linkages can also occur in this region but these are generally considered to be much weaker (Rasmussen, 1948). When this study was started no systematic investigation of steroidal olefines in this region had been recorded, but after our data had been collected, Bladon, Fabian, Henbest, Koch and Wood (1951*a*) reported a thorough analysis of band frequencies and intensities of relatively simple trisubstituted steroidal olefines, their reduction products and their isomers with tetrasubstituted ethylene groups. Although no overlap of band intensities was noted they cautioned that bands of intermediate strength might be diagnostically unreliable. However, they considered extreme weakness of a band in this region (790–850 $cm.^{-1}$) a dependable criterion for the absence and strong bands as a fairly safe indication for the presence of the trisubstituted olefinic linkage in the steroid molecule.

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The spectra of 3β-acetoxy-Δ^5-steroids with the usual configuration of the nucleus and without additional functional groups in the vicinity of the double bond present a very uniform picture between 790 and 820 cm.$^{-1}$. All showed two

FIG. 1

maxima near 800 and 812 cm.$^{-1}$. The higher frequency peak is relatively weak in those compounds that contain no polar group in the side chain or one rather distant from the nucleus but is rather strong in those possessing a polar group at or near ring *D*, as e.g. dehydro*iso*androsterone acetate. As these twin bands are not seen in their reduction products it seems justified to conclude that they are brought forth by the presence of the 5.6 double bond. Their position was constant within 1 cm.$^{-1}$ as long as compounds substituted in the 16 position were excluded. 16α-Oxygenation caused a slight but consistent shift of both bands to higher frequencies (803, 813–814) while diosgenin acetate, in which the substituent at C-16 is β oriented (Hirschmann *et al.*, 1949), absorbed at 797 and 814 cm.$^{-1}$. We must conclude therefore that vicinal effects can be detected if the site of substitution and the double bond are separated by 4 carbon atoms. While this distance may seem large it is no greater than that shown by Barton and Cox (1948) to give rise to optical anomalies under certain conditions. Above 820 cm.$^{-1}$, the spectra are far less uniform. Most of the peaks are rather weak, but a well defined maximum near 830 cm^{-1} occurs in several 17-oxygenated compounds including reduction products, such as *iso*androsterone and its acetate. These peaks therefore appear to be unrelated to the presence of the 5:6 double bond. (A contrary view was taken by Furchgott *et al.* (1946, 1947) concerning the maximum of dehydro*iso*androsterone at 831 cm.$^{-1}$ in carbon disulphide and at 835 cm.$^{-1}$ in the solid state.)

FIG 1. Infra-red spectra of carbon disulphide, 3β-acetoxystigmasta-5 22-diene, 3β-acetoxycholest-5-ene [illegible]
pregn-5-ene,
pregn-5-en-1[illegible]
en-17-one,
3β 16α 20α-tr[illegible]
one, 3β 16α [illegible]
[illegible]
[illegible]ent, unless indicated otherwise. The abscissa gives wave numbers in cm.$^{-1}$.

The effects of structural changes closer to the double bond are shown in Fig. 2. If an acetoxy group is introduced

[illegible] 3β 7β-diacetoxy- [illegible] methyl 3β- [illegible] 3β-hydroxy- [illegible] androst-5-ene [illegible] xypregn-5-ene, methyl 3β-hydroxychol-5-enate, [illegible] lest-5-ene, 3α-acetoxycholest-5-ene, and 3α-hydroxycholest-5-ene.

adjacent to the double bond no peak is observed near 800 cm.$^{-1}$. If the nuclear framework is altered the two bands are seen but their position is shifted. In dimethyl 3β-acetoxy-Δ^5-ætiobilienate (3β-acetoxy-16:17-*seco*androst-5-ene-16:17-dioate) the strain at the juncture of rings *C* and *D* has been released by cleavage. Diacetyldihydrojervine which contains a 5-membered ring *C* (Fried *et al.*, 1951) shows even larger departures from the normal pattern. Changes in the substituent at C-3 had little effect on the lower frequency band. In 3β-hydroxy and 3β-methoxy compounds (not shown) the second band appeared near 807 cm.$^{-1}$. In the absence of polar groups near ring *D*, it was very weak and in cholesterol it could not be seen at all. Since it is very desirable to base the diagnosis of bond position on the coincidence of more than one frequency it is preferable to examine in such cases the acetylated rather than the parent steroid. However, hydrolysis to the free compound with its concomitant spectral change can furnish valuable confirmation. The last two curves in Fig. 2 represent 3α-oxygenated steroids. Since the effect of epimerization at C-3 on the spectrum is profound it should be noted that the α configuration of Δ^5-unsaturated 3-acetates most probably can be recognized by observations near 1,240 cm.$^{-1}$ The stretching motions of the acyl-oxygen bond of the ester group result in a single maximum in 3β-acetoxy Δ^5-compounds (Jones *et al.*, 1951) and a triple peak (1,255, 1,243, 1,229 cm.$^{-1}$) in epicholesterol acetate. (See also Furst *et al*, 1952.) The spectra of 3β-chloro-Δ^5-steroids (Fig. 3) again are in accord with each other The curves are extended to 750 cm.$^{-1}$ to demonstrate another strong peak near 761 cm.$^{-1}$ which should greatly facilitate the recognition of chlorination in these compounds. The maximum shown by the reduction products near 805 cm.$^{-1}$ ought to caution against accepting any strong peak observed with an unknown steroid in this region as proof of a trialkylated double bond. Other anomalies of this type will be found in Fig. 4, which presents non-olefinic steroids and some with disubstituted or tetrasubstituted double bonds. None of these compounds is

expected to show strong absorption in this region and most of them indeed exhibit only very weak maxima. An exception is the rather strong peak of methyl 3α-acetoxychol-11-enate at 834 cm.[-1]. Less pronounced but still well defined peaks

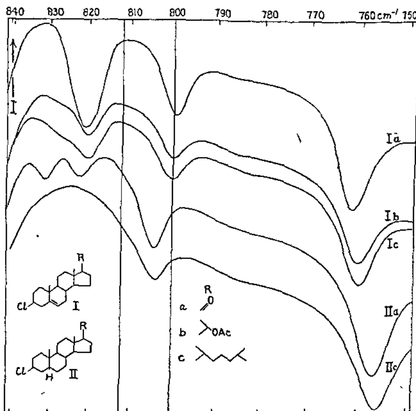


FIG. 3 Infra-red spectra of 3β-chloroandrost-5-en-17-one, 3β-chloro-20α-acetoxypregn-5-ene, 3β-chlorocholest-5-ene, 3β-chloroandrostan-17-one, and 3β-chlorocholestane.

are shown by *iso*androsterone acetate, tigogenin acetate, and 3β-acetoxycholest-6-ene. None of these compounds, however, exhibited the double peaks of the 3β-acetoxy-Δ^5-steroids.

A more rigorous test of the specificity of this pattern obviously requires the examination of the other trialkylated

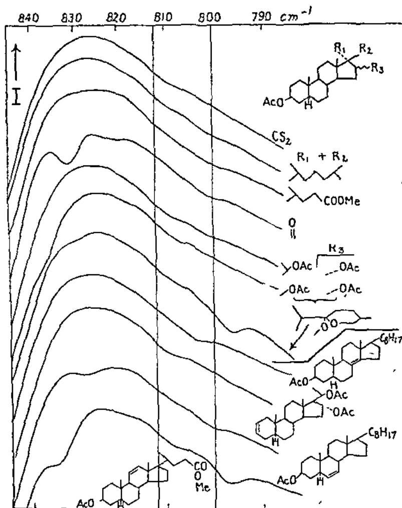
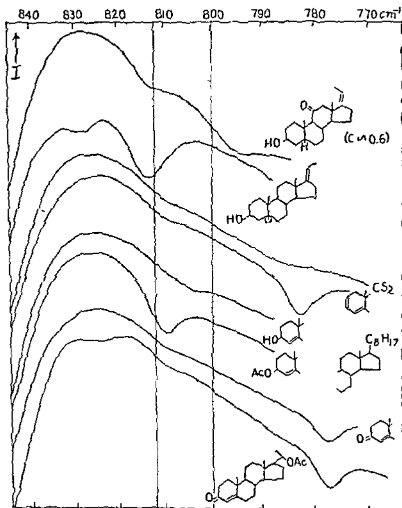


FIG. 4 Infra-red spectra of carbon disulphide, 3β-acetoxycholestane, methyl 3β-acetoxy-5α-cholanate, 3β-acetoxyandrostan-17-one,
17β-triacetoxyandro
acetoxycholest-8(14
3β-acetoxycholest-6

olefinic centres in the steroid molecule (Figs. 5 and 6). The spectra of the Δ^{14}-unsaturated compounds agree well with

that of "β"-ergostenol which was examined by the Manchester group (Bladon *et al.*, 1951*a*). The accord seems remarkable if



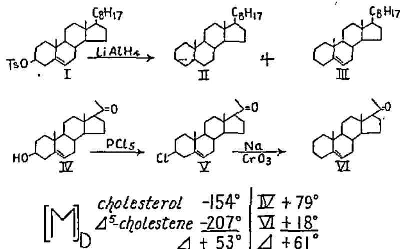
the close proximity of functional groups to the double bond is considered and suggests that all three peaks may possess diagnostic significance for the ethenoid linkage in this position. The five Δ^7-unsaturated compounds studied in the two laboratories are structurally less dissimilar. In the presence of a 3β-acetoxy group maxima were seen near 800, 829 and 846 cm.$^{-1}$. The $\Delta^{9(11)}$-unsaturated compounds showed a single major peak between 800 and 840 cm.$^{-1}$. The most likely cause for the shift of the maximum from its position in 3α-acetoxy-androst-9(11)-en-17-one (822 cm.$^{-1}$) and 3β-acetoxy-22a-5α-spirost-9(11)-ene (821 cm.$^{-1}$) to that in methyl 3α-acetoxy-eti-9(11)-enate (828 cm.$^{-1}$) is the configuration at C-5 since another compound with normal configuration at C-5, 24-hydroxychol-9(11)-ene, has been reported to absorb at 827 cm.$^{-1}$ (Bladon *et al.*, 1951*a*). *Cis* and *trans* 17-ethylenes are represented by only one example (Fig. 6) The spectrum of 3α-hydroxy-pregn-17(20)-en-11-one resembles those of Δ^5-3β-acetoxy steroids. If the same is true of its acetate, hydrolysis should permit spectrographic differentiation. Otherwise, no exception to the specificity of the twin bands of the Δ^5-3β-acetoxy-steroids has been encountered.

A simple Δ^4-unsaturated steroid, cholest-4-ene was reported by Bladon and co-workers (1951*a*) to absorb strongly at 810 cm.$^{-1}$. A very similar spectrum is given by 3β-acetoxy-cholest-4-ene, its parent hydroxy compound has only a weak peak at 805 cm.$^{-1}$ while cholestenone and cholestadiene showed little or no selective absorption between 790 and 845 cm.$^{-1}$. This phenomenon, however, appears not to be a general characteristic of conjugated systems since ergosterol, with two conjugated trialkylated double bonds, possesses two strong peaks at 801 and 836 cm.$^{-1}$ in carbon disulphide. It is possible, of course, that the olefinic carbon-hydrogen bending peak in cholestenone, for example, is not excessively weak but widely displaced. The maximum closest to the one seen in cholest-4-ene, appeared at 778 cm.$^{-1}$ and has its counterpart in the spectrum of 20α-acetoxypregn-4-en-3-one. Whatever the correct interpretation, we must conclude that very weak selective

absorption between 790 and 840 cm.$^{-1}$ does not exclude the presence of a trialkylated double bond if it is in conjugation or part of an allylic system and that the effect of structural changes at the carbon adjacent to the double bond can be profound.

The overlap of band intensities can pose real problems for the detection of trialkylated double bonds if the maxima can occur anywhere within the very wide range in which their hydrogen bending frequencies are commonly observed. The possibility of misinterpretation will be reduced if the trialkylated olefinic centres of the steroid nucleus can be characterized by absorption frequencies within narrowly defined ranges. To illustrate the advance which can be made by a more detailed analysis of band positions, I should like to present an example from the recent literature. Schmid and Karrer (1949) observed (I-III) that the reduction of cholesteryl toluenesulphonate with lithium aluminium hydride yields 3:5-*cyclo*cholestane and cholest-5-ene. When the reaction was repeated with the tosylate of pregn-5-en-3β-ol-20-one (Karrer *et al.*, 1951) only one pure substance was isolated after the keto group at C-20 had been restored by oxidation. It was believed to be pregn-5-en-20-one on the basis of its composition and the presence of a well established peak in the 12μ region. Comparison with the spectrum of cholest-5-ene, however, showed little similarity in this range. In particular the maximum near 800 cm.$^{-1}$ which has been observed in virtually all Δ^5-unsaturated compounds including cholest-5-ene was absent in the spectrum of the alleged pregn-5-en-20-one. As we had no reason to believe that the exchange of an *iso*octyl by an acetyl side chain could cause significant shifts of the Δ^5-bands, Dr. Daus undertook the preparation of pregnenone by the conventional route via the chloride. The authenticity and purity of the final product is indicated by its rotation which agrees well with the reference data of Barton and Cox (1948) and was confirmed by reduction to 5α-pregnan-20-one. Our preparation melted 23° higher than Karrer's product and its transmission spectrum was very similar to that of cholest-5-ene (Fig. 7).

The diagnosis of the location of trialkylated double bonds in the steroid nucleus by observations in the 12μ region suffers in its present state from two disadvantages, the occasional appearance of fairly strong bands unrelated to the presence of the double bond, and the rather large distances over which



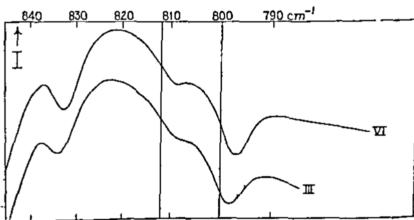


FIG. 7. Infra-red spectra of pregn-5-en-20-one prepared from V, and of cholest-5-ene. The rotations are for solutions in chloroform.

vicinal disturbances can be effective. Interference from unrelated peaks can be reduced by observing spectral changes upon reduction and other reactions The effect of neighbouring groups will require considerably more study which, however, may eventually repay in more detailed information about unknown steroids. With due regard to the possibility of such disturbances, however, the twin bands near 800 and 812 cm.$^{-1}$ should prove useful for the characterization of Δ^5-3β-acetoxysteroids The procedure is fast and simple, adaptable to a micro scale and, I believe in contrast to rotational methods and ultra-violet spectroscopy (Bladon, Henbest and Wood, 1951*b*), applicable to mixtures of moderate complexity such as may be encountered during the fractionation of natural products or in the course of synthetic reactions. The observations on steroids with other trialkylated double bonds are not yet numerous enough to warrant definite conclusions but hold promise of similar diagnostic possibilities.

[illegible]

[illegible]

substances contributed to this study Several compounds from the collection of the late Professor Schoenheimer were kindly made available by Drs D Shemin and D. Rittenberg This investigation was supported by grants from the Hanna Research Fund and the American Cancer Society on the recommendation of the Committee on Growth.

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(These references are incomplete. In particular, it should be noted that the spectra of several compounds studied have also been plotted by other investigators who have used various experimental conditions. Details on our experimental conditions and the locations of absorption maxima have been reported Hirschmann, H. (1952). *J. Amer. chem. Soc.*, **74**, 5357.)

DISCUSSION

CARDWELL: We have recently examined a large number of heart poisons which, from their very insolubility in CS_2 and other solvents [illegible] we have necessarily had to examine in

[illegible] only on a major band which, being absent in the parent hydroxy compound, is probably due to the acetate group. Thompson and

(about ± 2 cm^{-1}). While our observations are not sufficiently varied or

acetates it aids rather than complicates the diagnosis of the 5 6 double bond. It was chiefly for this reason that I recommended the use of

CARDWELL: In all the acetates we've examined so far, of the squill

differences and the ease of dehydration are much more diagnostic of that particular point

Dr. Strauss and I have also examined the infra-red spectra of the "α"- and "β"-anhydro-digitoxigenins and -digoxigenins. The "α"-anhydrogenins show no peaks in the 12μ region and are $\Delta^{8(14)}$ compounds.

BARTON: I would like to say how interested I was in the vicinal effects that Dr. Hirschmann reports.

the discrepancies that he reports in his spectra

H. HIRSCHMANN: I do not think we have sufficient data even to attempt a correlation between structure and the occurrence of infra-red shifts similar to Dr. Barton's analysis of molecular rotation anomalies. In our series, the interaction between a 16α substituent and the 5(6) double bond produced shifts of constant magnitude regardless of the nature of the substituent, which varied from an acetoxy group to a methoxy to a benzyloxy.

BARTON: The other point I'd like to ask you is this: you showed clearly that Karrer's compound wasn't the pregn-5-en-20-one. What do

spectrum but not by its rotation, I would not like to propose this structure without further evidence.

SOME ASPECTS OF THE STEREOCHEMISTRY OF C-20

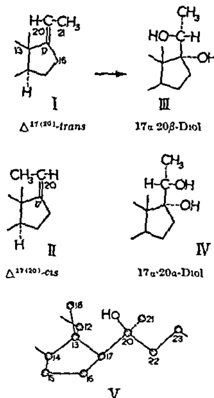
W KLYNE

My reason for discussing the stereochemistry of C-20 at a conference on adrenal steroids is that the 20-hydroxysteroids isolated from the adrenal cortex differ in configuration from most of the 20-hydroxysteroids isolated from other sources. Until recently there has been only one known instance of a pair of 20-epimers existing together in nature—viz. Reichstein's adrenal substances O and J (5α-pregnane-3β:17α.20α-triol and 5α-pregnane-3β:17α·20β-triol) (Reichstein, 1936; Steiger and Reichstein, 1938).

C-20 differs from all the nuclear carbon atoms in that its asymmetry is of the tartaric acid type, with free rotation about C-17–C-20 when this is a single bond. Any discussion of the stereochemistry of C-20 must begin from the work of Fieser and Fieser (1948, 1949) who first related C-20 to C-17. Their arguments are so familiar that I will only go over them briefly. (1) When a double bond $\Delta^{17(20)}$ is formed by dehydration of a 17-hydroxysteroid it is likely to give chiefly a *trans*-ethylene, with the methyl group (C-21) on the side of the double bond *away* from the *C* ring (I), and only a little of the *cis*-ethylene (II), where the C-21 methyl is crowded up against C-18 and C-12. (2) Hydroxylation of the *trans*-ethylene (I) by rear attack with osmium tetroxide must give III, a 17α:20β-diol. Hydroxylation of the *cis*-ethylene II would give a 17α 20α-diol IV. This argument provided the first correlation between C-17 and C-20 and has been generally accepted.

Lardon and Reichstein (1951) pointed out that perhaps the picture was not quite so simple, as in some of Sarett's work mixtures of 17:20-ethylenes had been obtained (Sarett, 1948, 1949).

Another line of evidence is available in the literature to support the Fieser configurations, and consideration of the stereochemistry required for elimination reactions would explain the anomalies to which Lardon and Reichstein drew

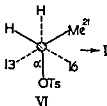


$\Delta^{17(20)}$-*trans* 17α 20β-Diol

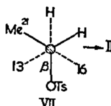
$\Delta^{17(20)}$-*cis* 17α·20α-Diol

attention (Klyne, 1951). The new evidence regarding C-20 comes first from work on the crystal structure of cholesteryl iodide by Carlisle and Crowfoot (1945). This showed quite definitely that the relationship between C-17, C-20, C-21 and C-22 in this compound (and therefore in cholesterol, cholic acid and related compounds) was as in V—or its mirror image. The side chain, C-22, etc., is thus α-oriented in terms of the

Fieser convention. Cholanic acids can be degraded to the 20-hydroxypregnanes *via* bisnorcholanic acids and norcholan-22-ones (Wieland and Miescher, 1949; Gallagher and Kritchevsky, 1950); (Meyer, 1951). Perbenzoic acid oxidizes ketones without rearrangement at the carbon atom where the hydroxyl group is introduced (Friess, 1949; Turner, 1950; Gallagher and Kritchevsky, 1950); therefore we should get a 20α-hydroxyl on oxidizing the normal 22-ketone. In fact we do; so this independent line of evidence supports the Fiesers' configurations. (Part of this argument has been suggested by Hirschmann and Hirschmann (1950), and it has been used in the reverse direction by Plattner (1951) to relate the configuration of the steroid side chain to that of the 20-hydroxysteroids.)



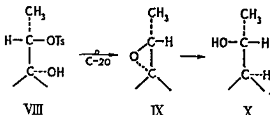
20α-Tosylate



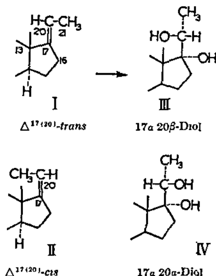
20β-Tosylate

Views looking from C-20 to C-17

—— bonds of C-20
- - - bonds of C-17

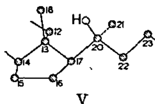


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$\Delta^{17(20)}$-*trans* 17α 20β-Diol

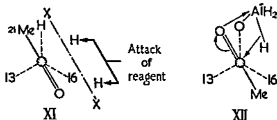
$\Delta^{17(20)}$-*cis* 17α 20α-Diol



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C-22 in this compound (and therefore in cholesterol, cholic
acid and related compounds) was as in V—or its mirror image.
The side chain, C-22, etc., is thus α-oriented in terms of the

which give largely 20β-hydroxy compounds are, first, platinum and hydrogen in acid (Marker *et al.*, 1937; Meystre and Miescher, 1946; Sarett, 1948, 1949). Here we can imagine the molecule settling on the surface of the catalyst as shown at X-X in formula XI (cf. suggestions by Linstead *et al.* (1942) in connection with hydrogenation of aromatic compounds). In the Meerwein-Ponndorf reduction with aluminium alkoxides (for mechanism see Woodward, Wendler and Brutschy, 1945; Doering and Young, 1950) the reagent needs plenty of space and presumably approaches from the direction of X-X Again the products of reaction are mainly 20β-hydroxy compounds (Marker *et al.*, 1941). Thirdly, lithium aluminium hydride in most cases gives a very high proportion of 20β-hydroxysteroid. This has been found with pregn-5-en-3β-ol-20-one, 5α- and 5β-pregnan-3β-ol-20-ones (cf. Klyne and Miller, 1950, Ott and Murray, 1948). If, however, lithium aluminium hydride is used to reduce a 17α-hydroxy-20-ketone (Hirschmann, Daus and Hirschmann, 1951; Brooks and Klyne, 1952), the products are chiefly 17α.20α-diols. I suggest that this is because the first action of the lithium aluminium hydride is to react with the free hydroxyl group at C-17 Then if the remains of this AlH_4 group react with the 20-ketone the latter must do so with ketonic oxygen pointing to the rear, giving 17α:20α-diol (XII). (Regarding the mechanism of reductions by lithium aluminium hydride see Trevoy and Brown (1949), Kenner and Murray (1950).)

There is only one reagent which in normal conditions gives a preponderance of 20α-hydroxysteroid—sodium and alcohol



XI XII

The allotment of configurations to the $\Delta^{17(20)}$ ethylenes formed from 20-hydroxy compounds by dehydration follows very simply from the need for *trans*-elimination in bimolecular elimination reactions (cf. Dhar, Hughes, Ingold, Mandour, Maw and Woolf, 1948; Barton, 1950). These dehydrations are carried out by heating 20-tosylates with tertiary bases. The 17α-hydrogen atom is removed by the base, and for an *E*2 reaction to occur, the 20-tosyloxy group must then be sticking forward as shown in VI (20α) or VII (20β). Formation of the double bond in the first case (20α) will give largely I (*trans*-ethylene; cf. Hirschmann, 1941). The 20β-tosylate gives largely III (*cis*-ethylene; cf. Sarett, 1948, 1949). A link between the 17:20-diols and the 20-hydroxy compounds not carrying an hydroxyl group at C-17 was provided by Sarett (1949) who obtained from a 17α-hydroxy-20α- tosylate VIII a 17α 20β-oxide IX, and then split this to give a 20β-hydroxy compound not carrying an hydroxyl at C-17 (X). Klyne and Barton (1949) almost simultaneously presented optical rotation evidence which is in agreement with these findings.

Conformations of the Side Chain

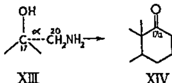
I want now to turn to a number of reactions which suggest that there are preferred conformations for a two-carbon side chain attached to C-17 by a single bond * (I shall consider only the normal 17β- side chain.)

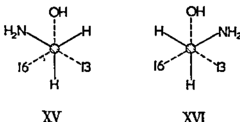
First let us consider reduction of a 20-keto group. In three cases where reduction is by hydrogen and catalyst or a reagent of large molecular size, the principal isomer formed is the 20β-hydroxy compound. These experimental facts appear to give some ground for assuming that 20-ketones tend to react in the conformation XI; this seems the least hindered conformation when Stewart models are considered. The reagents

* [illegible] important series of papers by Cram, D. J., [illegible]

field.

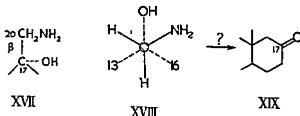
have shown that a quantitative yield of the 17a ketone is formed. Perhaps in the discussion someone can explain this.





Views from behind C-20 looking towards C-17.

In the case of the isomeric free 17β-aminomethyl-17α-hydroxy compound (XVII), however, the rearrangement involving the 16 17 bond seems likely to be severely hindered and we ought to get the 13.17 bond moving, to give the 17-ketone (XVIII → XIX).



I now come to the dehydration of 17·20-diols, which may ultimately have some direct bearing on the chemistry and the determination of adrenal metabolites. I was led to consider

(Marker *et al.*, 1937; Meystre and Miescher, 1946). This might be expected to give the isomer of lower energy content—as it does with cyclic compounds (cf. review by Barton, 1950). Whilst in nuclear positions the less stable polar hydroxyl groups are epimerized by heating with alkali, 20-hydroxyl groups are not (Marker *et al.*, 1937; Klyne and Miller, 1951, unpublished results on 20β-hydroxy compounds).

In the hydroxylation of a $\Delta^{20(21)}$ ethylene we find that 20α:21 and 20β:21-diols are formed in about the same proportions. Presumably when one group attached to C-20 is small, e.g. a hydrogen atom, we have no greatly preferred conformation.

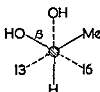
Rearrangements of 17 : 20-Disubstituted Steroids

We will next consider some rearrangements of 17:20-disubstituted compounds which lead or may lead to *D*-homo compounds by ring enlargement. One of the best known of these, the Tiffeneau reaction with 17-aminomethyl-17-hydroxy compounds (Goldberg and Monnier, 1940; Goldberg and Wydler, 1943; Prins and Shoppee, 1946), was often carried out with mixtures of starting materials obtained from mixed cyanhydrins and gave mixtures of 17a-ketones and 17-ketones of the *D*-homo series. Recently, however, Heusser, Herzig, Fürst and Plattner (1950) showed that it was possible to obtain the 17α-cyano-17β-hydroxy compound in a pure state and reduce this by lithium aluminium hydride to the 17α-aminomethyl-17β-hydroxy-compound (XIII); this, either in the free state or as its oxazolidine derivative with acetone, rearranges with nitrous acid to give the pure 17a-ketone (XIV). These pinacol type rearrangements require *trans* geometry between the heteroatom which is released and the carbon (with its electrons) which moves over to take its place. Now it appears from the models that in XIII the C-20-N bond can easily lie *trans* to the C_{13}-C_{17} bond XV or *trans* to the C-16-C-17 bond XVI. Rearrangements in these two positions would give 17 and 17a *D*-homo ketones respectively. In fact Heusser *et al.*,

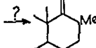
therefore seemed reasonable to postulate as a hypothesis that the urane derivatives might arise in the mare by rearrangement *in vivo* of 5α-pregnane-17α:20β-diols to 17-methyl *D*-homoandrostan-17a-ones—followed by reduction. Reference to the literature produced no proven examples of this reaction *in vitro*, but two examples which show there is much still to explain about the reactions of 17:20-diols. Steiger and Reichstein (1938) heated Substance J (5α-pregnane-3β:17α:20β-triol) (XXIII, R=H) with methanolic H_2SO_4 in the hope of obtaining the 20-ketone. This was formed, but could be isolated only after Girard separation in about 10 per cent yield. The non-ketonic fraction was not studied. Prins and Reichstein (1940) treated Substance K (5α-pregnane-3β:17α:20β·21-tetrol) (XXIII, R=OH) with HCl in boiling ethanol and obtained after acetylation a compound which they suggested might be a hindered ketone as it did not form a semicarbazone.

I have made preliminary experiments with the 5α-pregnane-3β.17α:20α(and 20β)-triols (Substances O and J), treating them with acid in the conditions used by Steiger and Reichstein (1938) and following this by Girard separation. Each of the two substances gives the 20-ketone, but in small yield (about 15 per cent). In the "non-ketonic" fraction each gives some unchanged triol (the 20β isomer gives about 30 per cent) and two compounds (one from each isomer) which from their chromatographic behaviour might be diols or hydroxyoxides. Infra-red examination (by Dr J. F. Grove and Dr. L. A. Duncanson of Messrs. Imperial Chemical Industries, Welwyn, Herts) shows that they are not hindered ketones. The compound obtained from the 20α-isomer in 45 per cent yield gives analytical figures in fair agreement with those required by $C_{21}H_{34}O_2$, $[\alpha]_D = -48°$ in chloroform, m.p. 179–182.° The compound obtained from the 20β-triol in 10 per cent yield has $[\alpha]_D = -50°$ in chloroform, m.p. 128–130°, and is not identical with that from the 20α-triol. Both these non-ketonic products give yellow colours with tetranitromethane and are therefore unsaturated Another non-ketonic compound obtained from

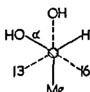
this problem by the peculiar nature of some steroids of pregnant mares' urine on which we have been working at Hammersmith (Brooks, Klyne, Miller and Paterson, 1952). Mares' urine differs from human pregnancy urine in that it contains urane derivatives, which we have shown are *D*-homosteroids (cf. XXI) (Klyne, 1950; Klyne and Shoppee, 1952), and also 20β-hydroxysteroids. Now as far as I know no 20β-hydroxysteroid has ever been reported from human urine, and urane derivatives have been obtained from this source only in traces. It therefore seemed worth speculating on a possible connection between urane derivatives and 20β-hydroxy steroids.



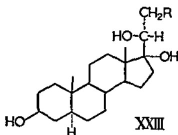
XX
17α.20β-Diol



XXI



XXII
17α 20α-Diol
Not favoured conformation



XXIII

A reasonable route for the formation of 17-methyl-17a-ketones of the *D*-homo series would be by a pinacol type rearrangement of 17:20-diols as previously discussed for the 20-amino-17-hydroxy compounds. Examination of Stewart models shows that only a 17α·20β-diol would be at all comfortable in the *trans* conformation (XX) required for this rearrangement (to XXI); a 17α:20α-diol would not (XXII). It

however, one 20β compound (5α-pregnane-3β:20β-diol) is a major constituent of the diol fraction, and another (5α-pregnane-3β:16α:20β-triol, Haslewood, Marrian and Smith, 1934; Hirschmann, Hirschmann and Daus, 1949) is present in the triol fraction.

Two 20-hydroxysteroids isolated from cows' bile (5α-pregnane-3β.20β-diol, Pearlman, 1944, 1946; pregnane-3α:20β-diol, Pearlman and Cerceo, 1948) are both 20β.

All the 20-hydroxy compounds so far isolated from adrenal extracts are 20β except for Substance O, which is 20α (For reviews see Reichstein and Shoppee, 1943, Heard, 1948.)

I hope that these somewhat speculative ideas on reactions at C-20 will provide material for discussion

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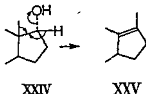
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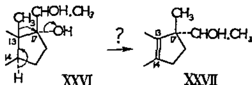
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the 20β-triol (in 35 per cent yield) has not yet solidified. So far there is no indication that the expected rearrangement of the 20β-triol to a 17-methyl-17a-ketone (XXI) occurs.

If I may offer a guess as to the nature of these unsaturated compounds obtained from the two triols, I would suggest that they might arise by a retropinacoline rearrangement involving C-17 and C-13 like that discussed by Miescher and Kägi (1949). These workers investigated the ψ-androstene derivatives obtained by acid rearrangement of 17α-hydroxy steroids not carrying an alkyl group at C-17 (XXIV); they suggested that these ψ-compounds were 17-methyl-13-nor-$\Delta^{13(17)}$ compounds of type XXV formed by a retropinacoline rearrangement. It may be noted that in XXIV the 13β-methyl and 17α-hydroxyl are antiparallel.





If the rearrangement products of the 17α:20α and 17α:20β-diols (XXVI) arise in a similar way they might be 17-methyl-13-nor-$\Delta^{13(14)}$- or $\Delta^{12(13)}$-steroids (XXVII or isomer). I hope to investigate this problem shortly.

Configuration of 20-Hydroxyl Groups in Natural Products

In all 20-hydroxysteroids of human urine the configuration is 20α (for review see Pearlman, 1948). In the pregnant mare,

KLYNE: Yes, I think it is quite possible. That's one of the reasons why I'm keen to go on with this work, because it does seem to provide us with some of the simplest reference substances to play about with.

MARRIAN: If that were so, then the rearranged compound would still have the α-glycol side chain, and should still be formaldehydogenic. Actually the 17-hydroxy adrenocortical steroids lose their formaldehydogenic properties on heating with acid.

KLYNE: Surely you would get quite a different picture with a ketone at C-20?

MARRIAN. Yes, I suppose you might

KLYNE I'm afraid at the moment I haven't considered all the possibilities that might arise there.

CARDWELL Is there no evidence in the infra-red of any hydrogen on that double bond in your compound XXVII?

KLYNE. No

CARDWELL: I presume that you haven't oxidized up to the ketone?

KLYNE. No

CARDWELL That would be a crucial test, you would not be able to

rated com-

KLYNE: No, I haven't

REICHSTEIN: What conditions did you use in the acid treatment?

KLYNE We used sulphuric acid in aqueous methanol, in exactly the same conditions as you used with Dr Steiger for Compound J, I deliberately chose the same conditions as you had used there

REICHSTEIN Do you know how strong it was?

KLYNE. About 5 per cent But when we've got more of the two triols, we'll certainly continue the work with different conditions

proximity of the two ester groups It was reasoned that the 20β-

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DISCUSSION

MARRIAN: I would like to start off with a theory myself. Have you considered the possibility that the known acid-instability of the 17-hydroxy cortical hormones might be due to a rearrangement similar to what you have just described?

KLYNE: Yes, I think it is quite possible. That's one of the reasons why I'm keen to go on with this work, because it does seem to provide us with some of the simplest reference substances to play about with.

MARRIAN: If that were so, then the rearranged compound would still have the α-glycol side chain, and should still be formaldehydogenic. Actually the 17-hydroxy adrenocortical steroids lose their formaldehydogenic properties on heating with acid.

KLYNE: Surely you would get quite a different picture with a ketone at C-20?

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CARDWELL: I presume that you haven't oxidized up to the ketone?

KLYNE. No.

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KLYNE No, I haven't

REICHSTEIN What conditions did you use in the acid treatment?

KLYNE. We used sulphuric acid in aqueous methanol, in exactly the same conditions as you used with Dr Steiger for Compound J, I deliberately chose the same conditions as you had used there.

REICHSTEIN: Do you know how strong it was?

KLYNE: About 5 per cent. But when we've got more of the two triols, we'll certainly continue the work with different conditions.

compounds we rather unexpectedly noted that the solvolysis rate of a 16α-acetoxy group seemed to depend very markedly on the configuration of the acetoxy group at C-20 The 16α-acetoxy group was readily attacked if the 20-ester had the β configuration but rather slowly if it had the α. This was interpreted as steric hindrance caused by the proximity of the two ester groups It was reasoned that the 20β-

There is another example which has puzzled some investigators and which can possibly also be explained on the basis of steric repulsion of the methyl groups at C-13 and C-20. I am referring to the different

chem. Soc , 64, 1655, Marker, R. E , Jones, E. M., and Krueger, J., 1940, *ibid.*, 62, 2532; Marker, R. E., and Rohrmann, E., 1939, *ibid.*, 61, 846) The nature of the stereoisomerism of these two types of furostan derivatives is not known but it must concern the configuration of either C-20 or C-22 or of both. It is very likely that C-20 is involved, since a

Dr. Klyne has put forward a very ingenious hypothesis to explain the high yields of 20α-hydroxysteroids that have been realized when certain 17α-hydroxy-20-ketones were reduced with lithium aluminium hydride. The concept, however, seems to need some modification to account for the reported failure of 3-ethoxy-17α-hydroxy-21-acetoxypregna-3 5-dien-20-one to yield pregn-4-ene-17α·20α:21-triol-3-one diacetate. If the proximity of another reacting group (21-acetoxy) is *responsible for this, it is not readily understood* why this compound was obtained in substantial (36 per cent) yield when (3-ethoxy-16α·17α-oxido-21-acetoxypregna-3:5-dien-20-one was subjected to the same sequence of reactions (lithium aluminium hydride, hydrolysis, and acetylation) (Julian, P. L., Meyer, E. W., Karpel, W. J., and Cole, W., 1951, *J. Amer. chem. Soc.*, 73, 1982.)

possible

tuted ethylenic bonds that Dr. Henbest has been investigating. With regard to your mechanism for $LiAlH_4$ reduction and the effect of the 17-hydroxyl group, this is somewhat analogous to the effect that we get in the acetylene series where we have an acetylene carbinol. Acetylene carbinols with a hydroxyl group α to the triple bond are reduced to give the *trans*-olefines, and the mechanism may be similar to the one operating here. You get the steric effect as the result of the formation of a complex.

Fieser. I might say that I have no strong opinion on this question. I only hope the matter is completely settled by the time we have to do our next book!

THE CHEMICAL ACTION OF X-RAYS ON SOME STEROIDS IN AQUEOUS SYSTEMS

JOSEPH WEISS

The chemical action of X-rays represents an entirely new attack on the steroid molecule. Before discussing this I will give a brief outline of the general principles underlying this work.

At first sight it may seem rather surprising that there exists no published work on the radiation chemistry of steroids prior to the present investigations, particularly in view of the great interest in the biological action of X-rays and the fact that the biochemistry of steroids has become very important, e.g. in relation to the problems of cancer. The reason for this is perhaps not too difficult to understand. Thus, while one possesses a perfectly good and clear theoretical basis for photochemical processes, provided, in the first instance, by Einstein's law of photochemical equivalence, such a principle was entirely lacking with regard to the chemical action of ionizing radiations. In fact, until relatively recently, the effects of ionizing radiations were considered to be purely "physical" in nature and their action on living tissues was looked upon as a destruction of certain sensitive "targets" in the cells by the bombarding particles (electrons, etc). Subsequently, when some chemical actions of ionizing radiations had become known it was assumed that specially "activated" molecules were formed, peculiar to these ionizing radiations, which did not occur under any other conditions (e.g. the hypothesis of the "activated water"). Thus, in a way it was a somewhat unrewarding task to study these chemical actions, which appeared to be outside the realm of ordinary chemistry. This belief was, in a sense, supported by some early experimental observations which seemed to show that in the case of

more complex organic molecules irradiation nearly always led to far-reaching destruction and degradations. On studying this problem (Weiss, 1944) we arrived at the conclusion that the processes of excitation and ionization, which had been well-known to physicists, should lead to relatively simple chemical changes. In fact, we were soon able to show that these radiations can produce well-defined chemical transformations even in relatively complex organic molecules, and this has led also to a new approach to the biological action of these radiations.

It is important to remember that, in spite of a certain parallelism, there is a very fundamental difference between the action of ionizing radiations (radiation chemistry) and photochemistry. In the latter case, the absorption of light is generally very specific and is connected with a certain molecular species, or often even with a definite bond system (as illustrated, for instance, by the well-known conversion of ergosterol into vitamin D_2). On the other hand, the absorption of ionizing radiations is non-specific, and is essentially a *mass effect*. Therefore, in dilute solutions, where there are a great number of solvent molecules and relatively few solute molecules, most of the primary processes induced by the radiations will take place in the solvent, where the greater part of the radiation is absorbed.

In the case of aqueous systems, which are of particular interest, we arrived (Weiss, 1944, 1947*a*, 1947*b*, 1949) at the conclusion that the processes of excitation and ionization eventually lead to a scission of water molecules according to the *net* process —

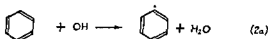


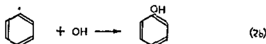
$$H_2O \rightsquigarrow H + OH \quad (1)$$

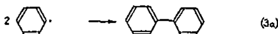
with the formation of hydrogen atoms and OH radicals.

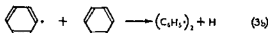
If this view is correct one should not only be able to bring about destructive oxidations (initiated, e.g., by OH radicals) but it should also be possible to carry out simple synthesis by introducing OH radicals into more complex molecules. This indeed proved to be the case: in the first instance we

carried out some experiments with aqueous solutions of benzene and were able to show (Stein and Weiss, 1948*a*, 1949*a*) that irradiation of these solutions by X-rays (with the exclusion of atmospheric oxygen) leads to the formation of *phenol* and *diphenyl*, which were isolated from the irradiated solution and fully identified by the usual methods. As the solvent here was the only source of oxygen this shows very clearly that the OH radicals must have been produced from the water, according to eqn. (1). Furthermore, the formation of diphenyl indicates clearly that one is dealing with a free-radical mechanism which can be briefly described by the following equations:—



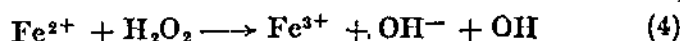






and which has been discussed more fully elsewhere (Stein and Weiss, 1948*a*, 1949*a*).

It is of interest to note that very similar reactions occur when benzene is allowed to react with OH radicals produced chemically, for instance, by means of the system hydrogen peroxide—ferrous salt (Fenton's reagent) where, according to Haber and Weiss (1934), the OH radicals are produced according to the reaction:—



$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH \qquad (4)$$

Before passing on to the discussion of steroids, I should like to mention that a more detailed study of the hydroxylation of benzene and of other simple benzene derivatives (nitrobenzene: Loebl, Stein and Weiss, 1949, 1950, phenol: Stein and Weiss, 1951; benzoic acid: Loebl, Stein and Weiss, 1951; etc.: Johnson, Stein and Weiss, 1951) has revealed a certain similarity between hydroxylation *in vitro* by OH radicals and the metabolic hydroxylation of these compounds, which has been studied particularly by R. T. Williams *et al.* (1950). For instance, one of the more striking facts is that the *m*-dihydroxy derivative (resorcinol) is formed neither in the biological oxidation of phenol nor in the oxidation by OH radicals (Garton and Williams, 1949). Further work has shown that the similarity between certain biological oxidations and those produced by OH radicals is not confined to these systems: we have found, for instance, that OH radicals can deaminate amino-acids (Stein and Weiss, 1948*b*, 1949*b*) and (in the presence of oxygen) this can lead to the formation of the corresponding keto acids (Johnson, Scholes and Weiss, 1951) (e.g. alanine is converted into pyruvic acid); a similar parallelism has been observed in a number of other cases.

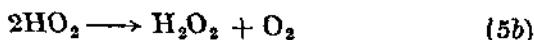
Whereas OH radicals are generally oxidizing, the hydrogen atoms which are formed simultaneously (eqn. 1) generally exhibit reducing properties. Thus, the above theory can account also for the previously known oxidizing and reducing actions of ionizing radiations in simple inorganic systems (cf. Lea, 1946).

In the presence of molecular oxygen it is well-known that hydrogen atoms react very efficiently according to:—



$$H + O_2 \longrightarrow HO_2 \qquad (5a)$$

leading to the formation of HO_2 radicals, which are themselves oxidizing radicals and which, under suitable conditions, can also lead to the formation of hydrogen peroxide according to:—



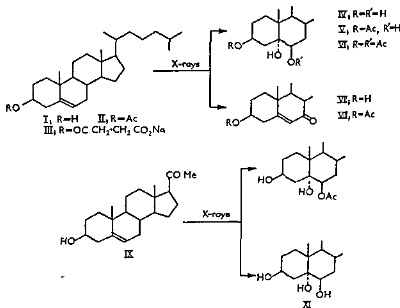
$$2HO_2 \longrightarrow H_2O_2 + O_2 \qquad (5b)$$

This reaction is generally responsible for the formation of hydrogen peroxide, which is often found in the action of X-rays on water.

In the experiments with steroids, reported below, we have always worked in the presence of oxygen so that we have generally only to consider oxidizing actions of OH and HO_2 radicals.

Cholesterol and 3β-Hydroxypregn-5-en-20-one

The first compound which we investigated in some detail was cholesterol (I) in aqueous solution (Keller and Weiss, 1950*a*, 1950*b*). This was accomplished by irradiating cholesteryl succinate (III) (sodium salt). On irradiation with X-rays (200 kV) with doses of the order of 10^6 r. we found that a definite chemical change was brought about, and we were able to isolate, by means of elution chromatography, a number of crystalline products. Our aim was, in the first instance, to establish with certainty the nature of the products

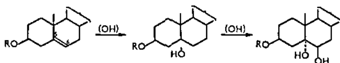


formed, and in all cases reported here we have isolated the reaction products, and all the crystalline products have been identified and fully characterized by the classical methods (melting point, derivatives, optical rotation, etc.).

In the case of cholesterol we found that the OH radicals attack the molecule, leading to (i) hydroxylation of the 5:6 double bond, and (ii) attack in the 7- position. Having thus established the products in purely aqueous systems we investigated solutions of cholesterol in aqueous acetic acid, where we found similar products, apart from certain acetyl derivatives. We then carried out some experiments on 3β-hydroxypregn-5-en-20-one (IX) (Keller and Weiss, 1950*b*), which gave results somewhat similar to those obtained with cholesterol, at least from a qualitative point of view. This is, of course, not surprising in view of the fact that these two molecules only differ in their side chains.

The experimental results regarding these two compounds are summarized in Table I and are briefly represented by the reaction scheme on p. 146.

The formation of the triol can be explained, in general, by the successive addition of hydroxyl radicals to the 5:6 double bond according to—

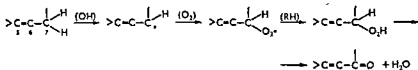


It is of some interest that we have found no evidence for the formation of an epoxide under the conditions of the experiments reported above. In fact, a preliminary study of this problem has led to the conclusion that the action of free radicals, such as OH (or CH_3COO) should lead directly to the dihydroxy or possibly to hydroxy-acetoxy compounds respectively, while primary epoxide formation generally seems to be the result of the attack by an ionic reagent such as CH_3CO^+ or HO_2^- on the (C=C) double bond.

IRRADIATION OF CHOLESTEROL AND PREGNENOLONE BY X-RAYS (200 kV)

Substance irradiated	*Solvent*	*Dose (approx.) in 10^4 r*	*Products*	*Yield per cent*
Na cholesteryl succinate (III), 0·25 per cent solution (in air)	Water	1·8	Cholestane-3β · 5α, 6β-triol (IV) Unidentified oil Starting material	19 ~15 50 } 84
Cholesteryl acetate (II), 0·17 per cent solution (in air)	Aqueous acetic acid (10 per cent of water)	1·8	3β · 6β-Diacetoxycholestan-5α-ol (VI) 3β-Acetoxycholestane-5α 6β-diol (V)	32 48 } 80
Cholesterol (I), 0·16 per cent solution (in air)	Aqueous acetic acid (10 per cent of water)	3·6	Cholesteryl acetate (II) 3β-Hydroxycholest-5-en-7-one (VII) Cholestane-3β · 5α · 6β-triol (IV) Unidentified oil Starting material	4·5 17·5 27·5 ~5 25 } 80
Cholesterol (I), 0·5 per cent solution (in a vacuum)	Glacial acetic acid	2·1	Cholesteryl acetate (II) 3β 6β-Diacetoxycholestan-5α-ol (VI) 3β-Hydroxycholest-5-en-7-one (VII) Unidentified oil Starting material	7 4 7 ~4 72 } 94
3β-Hydroxypregn-5-en-20-one (IX), 0·3 per cent solution (in air)	Aqueous acetic acid (10 per cent of water)	2·7	6β-Acetoxy-3β 5-dihydroxy-5α-pregnan-20-one (X) 3β 5α 6β-Trihydroxy-5α-pregnan-20-one (XI) Starting material	18 25 31 } 74

As shown in Table I in the case of cholesterol, as well as the triol, the 7-ketone has also been found. This we have explained tentatively by a repeated attack of OH radicals on the 7-position (possibly through the intermediate formation of a 7-hydroxy compound), while in the presence of atmospheric oxygen there is also the possibility of a reaction according to:—



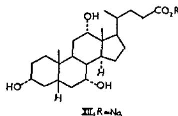
It appears, however, that in the relatively concentrated acetic acid solutions one must take into account also the reactions of (CH_3CO) radicals and possibly also of the CH_3CO^+ -ion, in order to explain the formation of the acetyl derivatives which have been found, although the detailed mechanism has not yet been fully established.

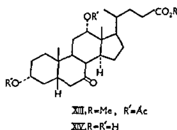
As will be seen from Table I, among the substances which have been found is cholestane-3β.5α:6β-triol, which may be of some interest since this compound has been isolated from arteriosclerotic aortas (Hardegger, Ruzicka and Tagmann, 1943) as well as from pigs' testes (Ruzicka and Prelog, 1943) and from beef liver (Haslewood, 1941). Likewise the 7-ketone appears to have been found in bulls' testes (Steinmann, 1943) and in pigs' testes (Prelog, Tagmann, Liebermann and Ruzicka, 1947)

Although relatively large doses of X-rays had to be employed in this work in order to isolate crystalline products, the relatively good yields and excellent recovery and the comparatively simple chemical changes involved, make it probable that essentially the same substances could be formed with very much smaller doses, as used under *biological conditions.*

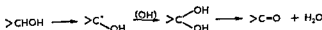
Cholic Acid (Keller and Weiss, 1951*a*)

An approximately 0·5 per cent solution of the sodium salt (XII) was irradiated with a dose of about 10^6 r of X-rays (200 kV). On elution chromatography after methylation of the crude product with diazomethane, about 80 per cent of the starting material was recovered; and of the two oily fractions which could be isolated one of the fractions (~ 7 per cent) gave a crystalline product after acetylation which was shown to be methyl 3α:12α-diacetoxy-7-ketocholanate (XIII). Hydrolysis of this product gave 3α:12α-dihydroxy-7-ketocholanic acid (XIV), which therefore must be the substance originally formed.



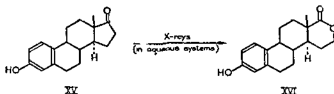


The formation of this substance is of some interest because this keto-acid appears as an intermediate in the biological oxidation of cholic acid by *Alkaligenes fæcalis* (Hohn, Schmidt and Hughes, 1944) and this demonstrates again that the action of OH radicals (e.g. produced by X-rays) is similar to the processes in certain biological oxidations. This observation may also shed some further light on the mechanism of the oxidative attack in the 7-position, as in the present case the transformation of the CH.OH group into the keto group should correspond to the following two stages:—



Œstrone (Keller and Weiss, 1951*b*)

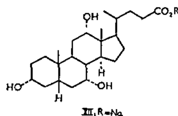
Œstrone (XV) was irradiated in (*a*) alkaline and (*b*) aqueous acetic acid solution, with X-rays (200 kV) in the manner described previously (Keller and Weiss, 1950*b*). In both cases we were able to isolate a crystalline compound, in a yield of about 3 per cent, corresponding to the formula $C_{18}H_{22}O_3$ (m.p. 311°–313°) which suggested that this substance was related to one of the lactones prepared previously by Westerfeld (1942) or Jacobsen (1947). Although the melting points given for these lactones were somewhat higher (334°–339°), when we repeated the procedure of these authors we found that the lactones thus prepared melted at 310°–312° (decomp.) (Kofler block) and that there was no depression of the melting point on mixing them or their acetates and also the optical rotations were the same in all cases ($[\alpha]_D^{21}$ + 39·8° ± 4°, in pyridine). Thus, it appears that these lactones are themselves identical, and also identical with the radiation product. Of

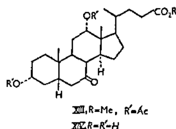


the two possible structures suggested by Jacobsen (1947) and Westerfeld (1942) respectively, we think that the former structure (XVI), is the more probable one for the reasons given previously (Keller and Weiss, 1951*b*). It is of interest to note here that there is no attack on the aromatic nucleus although in view of the results obtained with simple benzenoid compounds one might have expected some further attack of the aromatic ring. One is therefore led to conclude that the 5-membered ring is much more reactive towards OH radicals and thus, in a sense, the attack by the radicals shows a certain "specificity", as we also found in other cases.

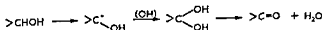
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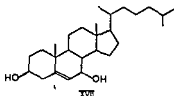


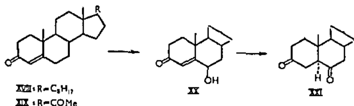


The formation of this substance is of some interest because this keto-acid appears as an intermediate in the biological oxidation of cholic acid by *Alkaligenes fæcalis* (Hohn, Schmidt and Hughes, 1944) and this demonstrates again that the action of OH radicals (e.g. produced by X-rays) is similar to the processes in certain biological oxidations. This observation may also shed some further light on the mechanism of the oxidative attack in the 7-position, as in the present case the transformation of the CH.OH group into the keto group should correspond to the following two stages:—



which had been obtained previously. The irradiation in methanol under conditions similar to those described previously (in the presence of air) gave a yield of about 5 per cent of 3β:7β-dihydroxycholest-5-ene (XVII).





Subsequently, we investigated the radiation chemistry of cholest-4-en-3-one (XVIII) which seemed of interest in view of the similarity of its skeleton to progesterone and other steroids of biological importance. In this case we were able to isolate one crystalline product from the irradiated solution which, on recrystallization from methanol (needles, m.p. 186°–192°), was identified as 6β-hydroxycholest-4-en-3-one (XX) (Ellis and Petrow, 1939) with m.p. 192°. It was further identified by preparing the semicarbazone. Refluxing the hydroxy compound in 90 per cent ethanol with a few drops of conc. HCl gave 3.6-diketocholestane (XXI) which on recrystallization from ether-petroleum gave needles m.p. 167°–171°, not depressed on admixture with an authentic specimen, kindly supplied by Professor V. Prelog. This compound showed only negligible absorption in the region 230–250 mμ. and was further identified by means of the dioxime.

On physico-chemical grounds this is not entirely unexpected: the reaction velocity is given by a general expression of the form:—

$$\text{reaction velocity} \sim A \, . \, e^{-E/RT}$$

where A is governed by the number of collisions and by a probability factor (which includes, e.g., steric effects), generally independent of the temperature, and where the energy of activation (E) enters exponentially. Thus, even a relatively *small* difference in the activation energies of two reactions may make a big difference in the corresponding reaction velocities and may lead to the result that the attack is practically entirely directed towards a particular centre of the molecule.

Other Steroid Hormones

Most of the work reported above has already been published in detail. I should like to discuss briefly some of our as yet unpublished work, carried out in collaboration with Dr. M. Keller and Mr. B. Coleby, relating to some other steroid hormones and related substances.

Experiments with these substances in aqueous media were on the whole not very successful because we were unable to isolate well-defined crystalline products, but we are hoping now to identify some of the compounds formed by means of paper chromatography. Subsequently we found that on irradiating these substances in methanol solutions crystalline products could be obtained.

At present very little is known about methanol as a solvent in radiation chemistry. It is very likely that OH radicals are again produced and we are at present engaged on a more detailed investigation of the irradiation of methanol itself.

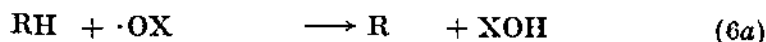
[illegible] results which we [illegible] of these steroids [illegible] :[illegible] d out again some experiments with cholesterol and here we were able to isolate 7-hydroxycholesterol as well as the triol and the 7-ketone

form and treatment even with dilute acids did not seem advisable in this case in view of the relatively labile side chain. We have, however, been able to isolate one crystalline substance from the irradiated solution of cortisone, which was identified as adrenosterone (androst-4-ene-3:11:17-trione). This could be formed by the oxidative splitting off of the side chain, but it is rather uncertain yet whether this is a genuine product of the irradiation.

The action of ionizing radiations on sterols, that is the attack of sterol molecules by free radicals produced, e.g., by the action of X-rays, represents a new and interesting field, particularly as we have been able to show that, in many cases, compounds are formed which are very similar to those formed in certain biological oxidations.

However, this does not necessarily imply that OH radicals are operative in biological oxidations although this is certainly not impossible in some cases in view of the fact that, e.g., the system hydrogen peroxide-catalase can bring about oxidation of a number of substrates under suitable conditions.

Although the actual rôle of OH radicals in biological oxidations is not known at present, the close parallelism between biological oxidations and the free-radical reactions suggests very strongly that many biological oxidations proceed by a univalent mechanism. This may be due either to an attack by a free radical (e g. of the type $\cdot OX$), by radical ions, or by suitable metal ion complexes (Me^{+n}. Compl.), which in general will also lead to a univalent attack. The general reactions relating to these processes may be represented schematically by the following equations:—



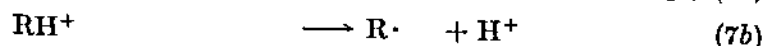
$$RH + \cdot OX \longrightarrow R + XOH \quad (6a)$$



$$R\cdot + \cdot OX \longrightarrow ROX \quad (6b)$$



$$RH + (Me^{n+}.Compl) \longrightarrow RH^{+} + (Me^{(n-1)+}.Compl) \quad (7a)$$



$$RH^{+} \longrightarrow R\cdot + H^{+} \quad (7b)$$

We also studied the action of X-rays on progesterone (XIX) in methanol. In this case we were not able to isolate a crystalline hydroxy compound but after isomerization in ethanol (with a few drops of HCl) we obtained 5α-pregnane-3 6.20-trione (XXI; $R=CO.CH_3$) which on crystallization from acetone-ether gave short prisms, m.p. 226°–230°, not depressed on admixture with an authentic specimen kindly supplied by Winthrop-Stearns Inc., Rensselaer, N.Y. Thus, in both these cases hydroxylation in the 6-position has taken place.

This is of particular interest because it has been shown recently that biological hydroxylation can also take place in the 6-position (Haines, 1952), and is not confined to the previously found attack in the 11-position. From the work of Ehrenstein (Ehrenstein and Stevens, 1940, 1941; Ehrenstein, 1941) it is known that the 6-position is normally the more reactive position of the molecule and this has been confirmed now by the attack by OH radicals, showing again the parallelism to the biolgical oxidation.

A number of experiments were directed towards the radiation chemistry of cortisone in solution. Our primary aim was to study the hydroxylation of cortisone and, if possible, to isolate some of the hydroxylated products, particularly with a view to studying their biological properties. The fact that very large quantities of cortisone are necessary in clinical use makes it not improbable that cortisone is only a precursor of a more active substance and it is not at all improbable that a hydroxylated product would be biologically more active and might correspond to one of the naturally occurring compounds.

Irradiation of cortisone in aqueous acetic acid solutions led to some attack but did not give any crystalline products. Irradiation in methanol gave also an attack on the cortisone molecule but again we found considerable difficulties in isolating any crystalline products. In view of our previous results on cholestenone and progesterone it is very likely that there was again an attack in the 6-position. However, the 6-hydroxy compound seemed difficult to obtain in a crystalline

the case of aqueous systems, for instance, free hydroxyl (OH) radicals are produced and their reactions with cholesterol, pregnenolone, cholic acid, and œstrone, have been reviewed and discussed. The products obtained by the action of these free radicals produced by the ionizing radiations are in many cases identical with those obtained in certain biological oxidations. This has been illustrated again by the more recent experimental findings that compounds such as progesterone, or cholest-4-en-3-one are hydroxylated in the 6-position. The importance and interest of the chemical actions of ionizing radiations lies in the fact that this may lead to a rational explanation of the biological effects of ionizing radiations on a biochemical basis.

I should like to express our grateful thanks to the Medical Research Council for the financial support of this work.

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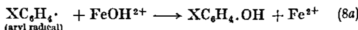


$$R\cdot + (Me^{+n}.Compl) \longrightarrow R^{+} + (Me^{+n-1}.Compl) \quad (7c)$$



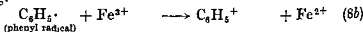
$$R^{+} + OH^{-} \longrightarrow ROH \quad (7d)$$

In fact, we have recently obtained some direct experimental evidence for the hydroxylation by metal ions and metal ion complexes as we were able to show (Stein and Weiss, 1951) that free phenyl or aryl radicals can be hydroxylated by means of ferric ions, e.g. according to:—

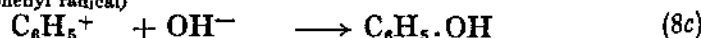


$$\underset{\text{(aryl radical)}}{XC_6H_4\cdot} + FeOH^{2+} \longrightarrow XC_6H_4.OH + Fe^{2+} \quad (8a)$$

and e.g.



$$\underset{\text{(phenyl radical)}}{C_6H_5\cdot} + Fe^{3+} \longrightarrow C_6H_5^{+} + Fe^{2+} \quad (8b)$$



$$C_6H_5^{+} + OH^{-} \longrightarrow C_6H_5.OH \quad (8c)$$

and these reactions are generally of some importance in the mechanism of the action of Fenton's reagent.

From an experimental point of view ionizing radiations represent an ideal means of producing free radicals in solution and they are thus very useful in the study of the attack of these radicals on biologically important molecules. From a more general point of view, the importance and interest of the chemical action of ionizing radiations lies in the fact that this may lead to a rational explanation of the biological effects of ionizing radiations on a biochemical basis, which has become increasingly apparent during the last few years (cf. the effect of molecular oxygen on chromosome breaking, etc.).

There is every reason to believe, therefore, that the study of the chemical action of ionizing radiations will contribute materially to the elucidation of problems in biological chemistry and radiobiology.

Summary

It has been shown that the chemical actions produced by the irradiation with X-rays of a number of steroids in dilute solutions are mainly due to the free radicals produced from the solvent under the influence of the ionizing radiation. In

WEISS: These reactions have not yet been investigated in detail. In fact, we did not particularly want to go over to the non-aqueous solvents, about which we know very little at present, but Dr. Keller

Yes, cholic acid.

FIESER: I suggest that you try introducing the hydroxyl at 11.

WEISS: Of course the activation energy would be very low there, and

HECHTER: I don't want to anticipate the discussion tomorrow, but I should mention that Dr. Harold Levy at the Worcester Foundation considers that a reasonable mechanism for the 11β-hydroxylation of steroids by adrenal enzymes might involve attack by a hydroxyl free radical.

WEISS: Of course I am very biased in favour of free radicals. One thing that surprised us very much is that when we start from phenol,

aqueous solution?

WEISS Not exactly—the yields were too low. But we did get the

allylic OH group, then oxidation is almost certainly due to attack by one or two radicals, probably hydroxyl radicals, at the allylic C-7

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DISCUSSION

in our laboratory worked up the mixture carefully by paper chromatography, and we examined it by UV absorption and infra-red, but it was essentially unreacted material Probably the question of conducting the reaction under proper conditions requires much more work.

WEISS: I think this is probably due to the solvent. We found that the solvent can make a lot of difference.

HECHTER: Dr. Weiss, when you worked with the corticosteroids did you try water as a solvent?

WEISS. I don't think that we ever did it in water. Cortisone isn't soluble in water, is it?

out, although there was a very good conversion.

HECHTER: How much of your steroid disappeared?

WEISS· It depends on how long you irradiate, but nearly all of it disappeared.

HECHTER: In how long?

WEISS. *That is a little technical, because it depends on the number*

PART II

METABOLISM OF ADRENOCORTICAL STEROIDS

BIOGENESIS OF ADRENAL CORTICAL HORMONES*

OSCAR HECHTER

If the answers to questions asked in science increase arithmetically, the new problems which arise as a result of these answers increase geometrically This variation on a theme by Malthus applies particularly to the field of corticosteroidogenesis. A few years ago the questions asked were simple and seemed relatively straightforward. Is cholesterol the precursor of the adrenal cortical hormones. And which of the some 28 steroids extractable from adrenal tissue are, in fact, secreted into the blood stream? Most workers felt that only a limited number of corticoids, plus an adrenal androgen, represented the adrenal secretory product. If one knew the starting material and the final end-products, the intermediate steps might be solved. ACTH stimulates biosynthesis of corticosteroids. Therefore it appeared not unlikely that one or more of the enzymatic systems giving rise to the chemical groupings which differentiated corticoids from other hormonal steroids, e.g., the ketol side chain, or C-11 oxy functions, would ultimately be shown to be regulated by ACTH. The work of the past few years has provided a basis for the beginning of an understanding of the biochemical mechanisms involved in corticosteroid synthesis. Many previous speculations

*The work described was aided in part by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army, under Contract No. DA-49-007-MD-255

position plus attack by oxygen at the double bond, leading to C-6
hain in corticosterone would
a hydrogen atom from the
y an hydroxyl radical at the
which this adrenosterone is
formed.

steroid ketols, identifiable by spot reactions, none of which appears to contain the Δ^4-3-ketone grouping generally believed to be necessary for biological activity in the corticosteroid field. We (Macchi, Zaffaroni, Pincus and Hechter, unpublished data) have uniformly found these unknown

Table I

THE α-KETOLS PRESENT IN ADRENAL PERFUSATES

α-Ketols	Micrograms per 2 liter samples of blood: Not perfused through adrenal	Adrenal Perfusates: No ACTH	Adrenal Perfusates: ACTH	Micrograms per 20 g beef gland extracts
Unknowns I–V	220	80	700	70
17-hydroxycorticosterone	360	145	1100	40
Cortisone	—[1]	25	200 (with Unknown VI)	20 (with Unknown VI)
Unknown VI[1]	—[1]	40		
Unknowns VII–IX	110	45	250	25
Corticosterone	400	230	1100	70
Unknown X	—[1]	60 (with Dehydrocorticosterone)	300	35 (with Dehydrocorticosterone)
Dehydrocorticosterone[1]	—[1]		250	
Deoxycorticosterone	120	35	140	—[2]

Note From unpublished data of Zaffaroni and Axelrod.

[1]Not detectable
[2]Not determined

ketols arising from isolated glands, perfused with five different ACTH preparations representing a diversity of products from several species. The appearance of these unknowns in the perfusate is independent of the dose level: minimal effective doses of all ACTH preparations evoke a pattern of ketol output qualitatively similar to that obtained with maximal levels of ACTH. Nor do these results appear to depend upon artifacts produced by a particular method of extraction. Qualitatively similar results are obtainable whether the perfusates are extracted with charcoal, dialysis or direct

based upon indirect studies have, in fact, been demonstrated by definitive techniques, but a host of unexpected complexities have arisen. These disturbances are to be welcomed, for when basic contradictions arise in science their solution generally brings the development of new basic concepts.

Today, I should like to review the field of corticosteroid hormone biosynthesis from a point of view where the unexpected findings are emphasized. I must add that much that I have to say is derived from discussions with colleagues at the Worcester Foundation (most particularly Drs. Gregory Pincus and Harold Levy), and thus represent concepts developed collectively by our group. I must take full responsibility for the unsupported speculation which will arise during the course of this discussion.

The Nature of the Adrenal Secretory Product

It now seems established that in *normal* adrenal glands, the major end-products of corticosteroid biosynthesis are two active hormones, corticosterone (Kendall's Compound B) and 17-hydroxycorticosterone (Kendall's Compound F). This is clearly the case in perfused isolated bovine adrenals (Hechter *et al.*, 1951), and appears to hold with perfused glands from other species tes [illegible] observations). [illegible] *in vivo* studies [illegible] adrenal venous blood samples, where compounds F and/or B likewise are the predominant components (Nelson, 1952; Bush, 1951). In addition to these specific steroids, a veritable galaxy of other compounds are simultaneously released into the circulation from perfused glands, albeit in smaller concentrations. Table I presents previously published data (Hechter *et al.*, 1951) in which the steroid ketols synthesized by the perfused bovine adrenal under the influence of added ACTH have been characterized by paper chromatography (Zaffaroni *et al.*, 1950). In addition to the large amounts of Compounds F and B, there is a minimum of 10 unknown

activity exhibited by corticosteroids. To cite but one of many possible examples: cortisone administered to the intact animal induces marked dissolution of lymphocytes, but is without significant influence on lymphocytolysis *in vitro* (Hechter and Johnson, 1949), whereas adrenal cortical extract is active under these conditions

It should be noted that the concentration of a particular corticosteroid released does not necessarily bear a relationship to the physiological importance of the substance involved. The secretion of trace amounts of highly active biological agents is fully as significant as the production of sizeable amounts of products such as Kendall's Compounds F and B. The physiological implications inherent in this picture, in which a multiplicity of corticosteroids are produced and released, brings us into the area of hormone action at a cellular and molecular level. Data in this area are meagre and speculation seems of limited value.

Initial Precursors of Corticosteroid Hormones

As reported previously (Zaffaroni *et al.*, 1951), the perfusion of radioactive cholesterol leads to the synthesis of radioactive Compounds F and B The basic rôle of cholesterol as a precursor of cortical hormones has thus been definitively established. C_2 fragments arising from acetate have been shown to condense to form cholesterol (Bloch, 1951). More recently, investigations with the perfused bovine adrenal (Zaffaroni *et al.*, 1951), and adrenal slices (Haines, 1952; Haynes *et al.*, 1952) have revealed that ^{14}C acetate likewise undergoes transformation to corticosteroids. Further analysis reveals that the situation is more complicated than $C_2 \rightarrow$ cholesterol $\rightarrow$ corticosteroids. Evidence has been presented that the utilization of 2-carbon fragments in corticosteroidogenesis does not necessarily involve cholesterol as an obligatory intermediary, and alternative pathways are clearly indicated (Hechter, 1952). This is not confined to corticosteroids; testicular formation of testosterone from acetate seems to occur without the participation of cholesterol as an obligatory intermediate

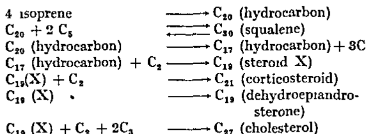
solvent extraction (either ethyl acetate or ether-chloroform, 4:1).

At first glance these unknown steroid ketols might appear to be a reflection of the abnormal *in vitro* conditions of the study, and hence of limited physiological interest. However, a study of the steroid pattern of adrenal venous blood taken from living animals clearly reveals the presence of components other than Compounds F and B (Nelson, 1952). While it is reasonable to assume that there may be quantitative differences between the *in vitro* and the *in situ* studies, and between various species, this should not obscure the basic qualitative similarity of the pattern which is emerging.

The chemical nature of the unknown steroid ketols under discussion is not established. They may include those described by Reichstein in his monumental studies characterizing the steroids extractable from adrenal tissue obtained at slaughter. Other products, however, are indicated as well. A variety of tetrahydro and possibly some dihydro derivatives of the 6 corticosteroid hormones containing the Δ^4-3-ketone grouping may be involved, as evidenced by spot tests on paper chromatograms. The biological activity in the corticosteroid series has generally been assumed to require the presence of the conjugated unsaturated grouping. This concept, however, merits critical re-examination in view of the recent finding of Grundy, Simpson and Tait (1952). These British workers report that the mineralocorticoid activity of adrenal extract *may largely be due to a steroid ketol, not possessing* the conjugated unsaturated system in ring *A*, which is perhaps 20 times more active than deoxycorticosterone (DOC) in salt and water metabolism. It may be noted that their studies are fully in conformity with the well-established finding that the activities of adrenal extracts are not explicable in terms of the known crystalline corticosteroid hormones. This would suggest that all corticosteroids derived from adrenal biosynthesis, or extra-adrenal metabolism, merit investigation of their possible biological activity, not in a single test, such as glycogen deposition, but over the full range of the diverse biological

The unique feature of this view involves the condensation of isoprenoid hydrocarbon to form the steroid nucleus with angular methyl groups in the correct positions.

I have already indicated that cholesterol is not an obligatory intermediary in steroid hormone synthesis. It thus seems not unreasonable to expect C_2 condensations to stop at a C_{21} or C_{19} stage. Retaining the idea that isoprene is an important intermediary, consider the following sequence.—



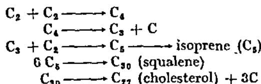
4 isoprene	$\longrightarrow$	C_{20} (hydrocarbon)
$C_{20} + 2\ C_5$	$\rightleftharpoons$	C_{30} (squalene)
C_{20} (hydrocarbon)	$\longrightarrow$	C_{17} (hydrocarbon) + 3C
C_{17} (hydrocarbon) + C_2	$\longrightarrow$	C_{19} (steroid X)
C_{19}(X) + C_2	$\longrightarrow$	C_{21} (corticosteroid)
C_{19} (X)	$\longrightarrow$	C_{19} (dehydroepiandrosterone)
C_{19} (X) + C_2 + $2C_3$	$\longrightarrow$	C_{27} (cholesterol)

This postulates condensation of isoprene to a 20-carbon hydrocarbon which, on losing 3 carbons at C-4, -13 and -14 (cholesterol nomenclature), condenses with a C_2 fragment to form a reactive C_{19} steroid. (Alternatively, $C_{20} + C_2 \rightarrow C_{22} \rightarrow C_{19} + 3C$). This reactive C_{19} steroid can be transformed to stable C_{19} steroids (e.g., dehydroepiandrosterone) or upon further condensation with active 2-, 3- or 4-carbon fragments, yields C_{21} or C_{27} steroids. To go from C_{19} or C_{21} to a C_{27} compound with the correct side chain of cholesterol, it is necessary to postulate $C_{21} + 2C_3$ type condensations. It should be emphasized that the entire sequence outlined, except for the squalene evidence and Bloch's suggestive evidence for isoprene condensation, is merely an exercise in arithmetic. I should like, however, to mention a finding which may be of some importance.

In a single experiment where non-labelled dehydroepiandrosterone (DHA) was perfused simultaneously with [^{14}C] acetate, a small amount of 17-hydroxycorticosterone was obtained which appeared to contain most of the radioactivity in the

(Brady, 1951). This state of affairs in the adrenal seems very reasonable. The adrenal gland responds almost instantaneously to various stresses with an outpouring of adrenal hormones; the desirability of having a store of precursor available for these reactions is obvious. Simultaneously, the gland has an apparatus for cholesterol synthesis from C_2 fragments that can be diverted from the pathway leading to the C_{27} storage form, to produce the C_{21} steroids needed. The picture described is analogous to carbohydrate metabolism, where glucose may be used directly through the glycolytic sequence or may be first converted to the storage form, glycogen.

If the assumption is made that 2-carbon fragments condense to form a reactive structure, designated as X, which has the potentiality of being converted to both cholesterol and corticosteroids, it is tempting to extend the speculation to involve X as a precursor for both C_{19} (androgens) and C_{18} derivatives. This raises the question of the specific intermediaries between C_2 and X. It seems generally agreed that the first step involves $C_2 + C_2 \rightarrow C_4$ (Gurin and Brady, 1951; Bloch, 1951), although it is not clear from their data why acetate should be a more efficient precursor than acetoacetate (or other 4-carbon intermediaries) assumed to arise as the initial product of the condensation. Beyond this, however, there is only speculative evidence to point the way. Bloch (1952) has speculated that C_5 units arising from C_2 (Bonner and Arreguin, 1949) form isoprene, which then polymerizes to form squalene, a C_{30} hydrocarbon. In support of this thesis, Bloch (1952) and his collaborators have been able to show that labelled squalene is transformed to cholesterol, without involving squalene degradation to C_2 fragments. This sequence is summarized below:—



Metabolic Pathways from Cholesterol to Corticosteroids

Knowing that cholesterol is a precursor, and that Compounds F and B are end-products, it becomes possible to envisage the type of reactions necessary to achieve this transformation. On the basis of studies in which various steroid substrates were perfused through bovine adrenals with sub-

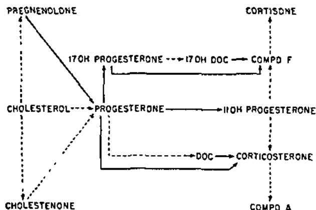


Fig. 1. Tentative scheme of [illegible]

as yet been demonstrated.

sequent isolation of products, it became possible to evaluate the enzymatic capacities of adrenal tissue to effect specific reactions. The basic findings have been previously published (Hechter *et al.*, 1951). The tentative scheme of corticosteroid biosynthesis presented by us in 1950 (Fig. 1), has undergone remarkably little modification as a result of subsequent work, but there are a few additions. Drs. R. W. Jeanloz and H. Levy (at the Worcester Foundation) have found that 11β-hydroxyprogesterone, considered as a possible intermediary in the synthesis of corticosteroids, is not converted to either F or B in

side chain (Hechter, 1952). Such a result would be consistent with C_2 condensation on a reactive C_{19} steroid acceptor formed from dehydroepiandrosterone. Recent experiments involving perfusion of ^{14}C labelled dehydroepiandrosterone (obtained from Dr. Ralph Dorfman) in two consecutive experiments have yielded small quantities of Compound F which, after repeated paper chromatography, contain demonstrable radioactivity.

The details of one of these experiments follow: 10 mg. of ^{14}C labelled DHA (2,600 counts per mg. per minute) was perfused through a beef adrenal for 3 hours. On paper chromatography of the perfusate, an "F" spot was obtained which, following two additional fractionations on paper, gave a homogenous spot by the spotting reactions available to us. It was eluted and contained the usual contaminants extracted from papers. By blue tetrazolium test the presence of 0·41 mg. of "F" equivalent was indicated; this material counted 310 counts per mg. "F" equivalent. It should be pointed out that a similar isolation of a spot which appears to be 11-hydroxyandrostenedione gave only 700 counts per mg. per minute. Dorfman has isolated the latter product in pure form from ^{14}C-DHA perfusates. The crystalline product he obtained had an activity equivalent to the DHA added. From this and previous work, we have come to regard counts of contaminated steroids as having little quantitative significance; accordingly the dilution in activity suggested should not be taken too literally. For that matter, the evidence that the radioactivity is due to "F", while strongly suggestive, cannot be regarded as definitively established until further work is done. Until a pure "F" derived from DHA is obtained and the ratio of specific activities compared quantitatively, these data should not be taken to indicate that a C_2 condensation actually occurs. *We hope to pursue these studies further*, isolating adrenal cholesterol, fatty acids, and other products which appear in the DHA perfusates to test aspects of the completely speculative scheme advanced here.

As it now stands, our scheme represents a series of deductions, based primarily upon the ability of substrates to react. Biochemical research has revealed innumerable examples of *in vitro* reactions, known to be relatively unimportant in normal metabolic pathways. Further work both at the *in vitro* and *in vivo* levels is clearly required to answer this very real limitation of our reaction sequence. This scheme, which will undoubtedly be modified as work proceeds, represents only a beginning in an attempt to chart metabolic pathways in steroid hormone biosynthesis. To this extent it is useful.

It is interesting to note that a comparison of the side chain of progesterone with the side chains of the end-products (F and B) indicates a superficial analogy to pyruvate and the trioses involved in glycolysis, respectively. As a matter of fact, if one considers possible reaction mechanisms for 17- or 21-hydroxylation, it is amusing to note that a reversal of the glycolytic sequence, starting from pyruvate, might afford a basis for the oxidative processes. If corticosteroids represent substituted trioses (with a very large R), all of the reaction possibilities involved in glycolysis come to mind. Perhaps the real "intermediaries" are phosphorylated steroids rather than the free alcohols we have indicated.

Another deficiency in our scheme involves cortisone and 11-dehydrocorticosterone (Kendall's Compound A); these are shown as derived from the oxidation of F and B respectively. It should be noted that neither reaction has been demonstrated directly, either in the perfused gland or in adrenal homogenates. In the absence of definitive information about the chemical structures of the unknown steroid ketols released by perfused glands, it is of course not possible to incorporate them into the reactions of corticosteroidogenesis. Eventually, however, they must be fitted into the sequence.

Action of ACTH in Corticosteroidogenesis

In a previous paper (Hechter *et al.*, 1951) I have considered the point of ACTH action in the reaction sequence starting from cholesterol. On the assumption that the pathways postu-

significant amount, although other products, of unknown structure, are indicated. Similarly, when [^{14}C] cholestenone (30,000 counts per mg. per minute) is perfused through glands for three hours, with and without added ACTH, although sizeable amounts of corticosteroids are produced with ACTH, the F and B spots eluted following paper chromatography do not contain measurable radioactivity. This would appear to exclude cholestenone from the pathway, and suggests that the removal of the side chain ($C_{27} \rightarrow C_{21}$) precedes the subsequent oxidation at C-3 to form the Δ^4-3-ketosteroid grouping. Starting from Δ^5-pregnenolone, a series of reactions are indicated in Fig. 1 involving progesterone transformation through separate pathways into 17-hydroxy- and 17-deoxycorticosteroids.

From pregnenolone on, all of the reactions indicated have now been achieved in adrenal homogenates. The conversion of pregnenolone to progesterone (Samuels, private communication); of progesterone to corticosterone, and of 17-hydroxyprogesterone to Compound F (Hayano and Dorfman, 1952); the 17-hydroxylation of progesterone to form Reichstein's S and Kendall's F (Plager and Samuels, 1952); as well as the 11-hydroxylation of DOC and Reichstein's S to form B and F respectively, have all been reported. Since this area will be covered by other participants in this conference, it is sufficient to state that all of the *in vitro* homogenate studies published to date are in full agreement with the scheme derived from perfusion data.

While this seems a satisfactory state of affairs, mention must be made of several basic weaknesses of the postulated scheme of corticosteroidogenic pathways. The major weakness perhaps is the postulation of pregnenolone as intermediary

with [^{14}C] acetate) with blood containing non-labelled Δ^5-pregnenolone as a trapping agent, we hope to obtain evidence on this point.

As it now stands, our scheme represents a series of deductions, based primarily upon the ability of substrates to react. Biochemical research has revealed innumerable examples of *in vitro* reactions, known to be relatively unimportant in normal metabolic pathways. Further work both at the *in vitro* and *in vivo* levels is clearly required to answer this very real limitation of our reaction sequence. This scheme, which will undoubtedly be modified as work proceeds, represents only a beginning in an attempt to chart metabolic pathways in steroid hormone biosynthesis. To this extent it is useful.

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In a previous paper (Hechter *et al.*, 1951) I have considered the point of ACTH action in the reaction sequence starting from cholesterol. On the assumption that the pathways postu-

lated mirror the actual processes involved, the deduction was made that ACTH does not appear to be fundamentally concerned with the reactions between pregnenolone and corticosteroids, but in an earlier step in the process, perhaps involving the degradation of the cholesterol side chain. Such a view in effect postulates that the enzymatic activities involved in the elaboration of the specific characteristics of corticosteroids are not regulated by ACTH. The difficulties which arise from placing ACTH action at an early point in corticosteroidogenesis, where the postulated reactions cannot be regarded as peculiarly specific for the adrenal cortex (e.g., cholesterol degradation to pregnenolone) have been previously discussed (Hechter, 1952). At the 1951 Macy Foundation Conference on the Adrenal Cortex I advanced some tentative speculations on ACTH action in which I postulated that its specificity resulted from a specific affinity of ACTH for adrenocortical cells. From this point, assumption was built on assumption, to finally yield a working hypothesis that ACTH acted on the cell surface, where it influenced ion permeability. This in turn altered cellular conditions so that the reaction cholesterol to pregnenolone was initiated.

As a result of the discussion which ensued, and the opportunity for retrospective thinking, I am now convinced that the view I presented at that time is too narrow to satisfactorily explain ACTH action. As Dr. C. N. H. Long emphasized, one must provide a mechanism by which ACTH stimulates protein synthesis in the adrenal as well as corticosteroid synthesis (on the assumption that there is a single ACTH). *Certainly ACTH must be considered as possibly acting in the corticosteroidogenic reaction sequence starting from acetate, whereas our own data suggest that cholesterol need not be involved. Finally, the mechanism by which ACTH increases* phosphate uptake and turnover in the adrenal (Gemzell, 1948) must be incorporated in any theory which purports to explain the mechanism of ACTH action.

I must add that our original deduction, that ACTH acts at an early step in corticosteroidogenesis, has now been confirmed

by recent perfusion studies done in collaboration with Dr. David Stone. On perfusing [14]C labelled cholesterol through adrenal glands in the presence and absence of added ACTH, the total percentage of administered radioactivity which was incorporated into the F and B isolated was 0·49 per cent with ACTH, and 0·02 per cent in the absence of ACTH. Not only were the absolute amounts of F and B increased, but the specific activities were increased four-fold with added ACTH. This is a 25-fold increase in the reactions from cholesterol, produced by ACTH. When similar experiments are done with [14]C labelled progesterone, our preliminary results, in three experiments indicate that ACTH only increases the incorporation of radioactivity into corticosteroids by a factor of some 20–25 per cent as compared to glands perfused without ACTH. In accord with our previous speculation, the conclusion now seems inescapable that ACTH does not significantly influence the reactions from progesterone to corticosteroids.

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DISCUSSION

Marrian: In your paper chromatograms of the perfusates from the glands, have you thought of using lead tetra-acetate for spotting in order to pick up compounds other than α-ketols? It is a useful reagent for this purpose

[illegible]

the very large amounts of dehydro*iso*androsterone in the urine of many patients with adrenocortical tumours. The formation of 17-ketosteroids [illegible]

dehydro*iso*androsterone were an intermediate in the synthesis of C_{21} compounds—an intermediate that is produced by tumour tissue in larger amounts than can be utilized. We tried to test this concept of the [illegible]

not necessarily mean little conversion, as we do not know the dilution of the injected dehydro*iso*androsterone by material synthesized *in vivo*. Unfortunately, the woman excreted so little of this compound that we were unable to analyse it. I am very glad to learn that there is perhaps some reality to this concept of a condensation reaction.

Hechter: What I have presented should be regarded as only preliminary. I want to re-emphasize that we have not as yet established definitively an actual C_2 condensation on a C_{19} steroid acceptor. If later such a reaction is established, I would feel that the reactive acceptor would probably not be dehydro*iso*androsterone, since it seems to be too unreactive for this rôle

Bush. I know that two of your unknowns I–V in adrenal perfusates have been tentatively identified as the 3-hydroxy analogues of Compounds E and F, and I wondered whether any of the others have been identified.

HECHTER: Our ACTH perfusates were turned over to Dr. Zaffaroni, who was then at Rochester. He separated the steroid ketols by paper

they were based only upon paper chromatography behaviour and sulphuric acid chromogens.

FOLLEY: I understood you to say that you perfused adrenals with [^{14}C]-acetate and then isolated cholesterol and Compound F from the perfusion fluid. You found that the specific activity of the Compound F was higher than that of the cholesterol, which suggested to you that cholesterol was not an obligatory intermediate. In this connection I would like to mention the work of Zilversmit, Entenman and Fishler (*J. gen. Physiol.*, 1943, 26, 325) who examined theoretically the time-relationships of the specific activities of a precursor substance which is being labelled in an organ and of its immediate reaction product. They deduced that the specific activity curve for the precursor will rise to a maximum and then decay exponentially, while that for the reaction product also rises but remains below the percursor curve until it, in turn, attains its maximum, when the two curves cross. The specific activity curve for the reaction product thereafter decays exponentially but at a higher level than the precursor curve. It is therefore possible that, under certain conditions, the specific activity of your reaction product could be higher than that of the precursor, depending on the time relationships. Of course, as I say, I don't know enough about your experimental conditions to know whether this could be a likely explanation of your results.

HECHTER: We certainly appreciate that point and recognize that

terol pool" in the adrenal? One could envisage a variety of adrenal cholesterol compartments cholesterol associated with Protein 1, Protein 2, etc , and esterified cholesterol also associated with various proteins. Now of these various cholesterol fractions only one might primarily be involved predominately in corticosteroid biosynthesis. Our method of fractionation for adrenal cholesterol separated it only into two major fractions, free and esterified But these in turn, as I have indicated, may be complex mixtures derived from various cholesterol pools. So to that extent our data might be interpreted as not necessarily being definitive against the idea of cholesterol as an obligatory intermediate in corticosteroidogenesis.

STUDIES OF THE ENZYMES INVOLVED IN THE SYNTHESIS AND DEGRADATION OF THE HORMONES OF THE ADRENAL CORTEX

LEO T. SAMUELS

THE work of Hechter, Pincus and co-workers has proved that the isolated adrenal gland can synthesize the active steroids of the adrenal cortex from such simple molecules as acetate. It also seems highly probable that these active compounds undergo changes which reduce their biological potency before they are eliminated from the organism; the rate of secretion of such substances in the adrenal vein blood, as measured by Vogt, Nelson, Bush and others, is much higher than the amounts which are found unchanged in the excreta. In the present studies an attempt has been made to throw some light on the mechanism by which these changes take place.

Because of its high concentration in the tissues that form steroid hormones, cholesterol has for a long time been thought to be a precursor of these compounds. The rapid drop in cholesterol following the stimulation of the adrenal gland by ACTH lends support to this concept. That this common sterol can be converted by the gland into adrenal hormones has been demonstrated by Hechter and co-workers (Hechter, 1952) perfusing ^{14}C-labelled cholesterol and identifying the isotope in hydrocortisone (Compound F of Kendall). Their yields, however, were smaller than those from acetate, and the hypothesis was advanced that both compounds were converted into some common intermediate before being formed into the active compounds. In our work, we have not been able to demonstrate the conversion of cholesterol to adrenal hormones by homogenates under the conditions used. We have, however, been able to convert a possible

intermediate which occurs in the gland, pregn-5-en-3β-ol-20-one, into these compounds by a series of enzymic reactions, the details of some of which have been worked out by Sweat (1952), Hayano and Dorfman (1952), and the Utah group (Samuels, *et al.*, 1951; Plager, 1952).

It is notable that those tissues forming the non-benzenoid steroid hormones all contain 3β-hydroxysteroids as well. With the identification of testosterone in the spermatic vein blood of dogs and after the perfusion of human testes, it appears well established that all non-benzenoid hormones which are secreted contain the αβ-unsaturated ketone structure in ring *A*. It is not surprising, therefore, that we have been able to demonstrate the conversion of Δ^5-3β-hydroxy compounds into Δ^4-3-ketosteroids by the corpus luteum, placenta, interstitial cells of the testis, and adrenal cortex (Table I). A wide range of other normal tissues was tested but no significant activity was found.

Table I

TISSUES WHICH CONTAIN THE ENZYME OXIDIZING RING *A* TO AN αβ-UNSATURATED KETONE

Tissue	*Substrate*	*αβ-Unsat ketone formed µmoles/g*
Cryptorchid testes, rat	Pregn-5-en-3β-ol-20-one	0 99 (0 93–1 02)
Corpus luteum, cow	,,	3 00 (2 04–4 02)
Adrenal cortex, cow	,,	2 23 (2 22–2 43)
Placental tissue, rat	,,	0 57 (0 54–0 60)
Cryptorchid testes, rat	Dehydroepiandrosterone	0·96

The specific nature of the hormones originating from the different tissues does not seem to be associated with any distinction between the enzyme systems catalysing this reaction. Thus interstitial cells produce progesterone from pregn-5-en-3β-ol-20-one and androst-4-ene-3:17-dione from dehydroepiandrosterone (androst-5-en-3β-ol-17-one). The

same is true of the adrenal cortex and, insofar as it has been tested, of the corpus luteum and placenta. Those characteristics of the system which have been compared in the different tissues have not differed significantly. It seems highly probable, therefore, that the same enzyme occurs in all four.

Diphosphopyridine nucleotide (DPN) appears to be a specific hydrogen acceptor for the system. No other cofactor tried, with the exception of ATP, influenced the rate of the reaction. ATP significantly affected the rate in the case of homogenates of the adrenal cortex but had no effect in the presence of testis homogenates. At first glance this would seem to belie the previous statement regarding the identity of the enzyme system in the different tissues. As will be shown, however, under the conditions of our incubation the reaction reaches equilibrium at approximately 1 mole pregnenolone to 4 moles progesterone, and the mass law effect causes significant variations from linearity at the levels of conversion achieved by the homogenate alone. Sweat (1952) and Hayano and Dorfman (1952) have shown that ATP activates the oxidation of carbon-11 by adrenal homogenates, an enzyme system which the testis does not possess. In the presence of ATP, therefore, progesterone is removed from the system by further oxidation and equilibrium is not approached. This accounts for the influence of ATP on the reaction in the presence of adrenal homogenates.

Failure to recognize that, under the conditions used, equilibrium was achieved while significant amounts of reduced substrate still remained in the system led to considerable wasted effort. It was assumed that the reaction would go practically to completion like the DPN-activated enzyme reaction in liver tissue which oxidizes the alcohol group on carbon-17 of C_{21} compounds to a ketone. Since concentrations of tissue similar to those used in the liver studies gave conversions significantly short of complete oxidation it was thought that the activity was similar. When, however, an enzyme concentration curve was run with adrenal homogenate, it was found that the activity was much greater, and

that the similarity of recovery in many of our previous studies was due to the reaction reaching equilibrium (Fig. 1). The same equilibrium appears to be achieved with testis interstitial cell tissue homogenates. When dehydroepiandrosterone is used as substrate, the equilibrium point is the same within the limits of the method, although it is achieved more

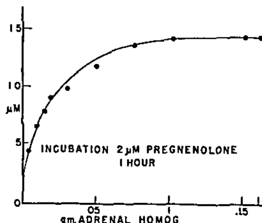


FIG. 1 The effect of increasing amounts of adrenal homogenate on the oxidation of pregnenolone to progesterone.

slowly. The same equilibrium is approached if the concentration of enzyme is kept constant and time is varied.

After recognition of the importance of the equilibrium in estimates of activity, studies of the activity of various tissues were carried out in which conversion never exceeded 50 per cent of the original substrate If this enzyme system plays a key rôle in the formation of the hormones, such values should bear some relation to the relative output of hormones by the particular organ. In Table II the activity in the adrenals of various species is given, expressed as micromoles pregnenolone oxidized per gram tissue per hour. The adrenals of rodents are much more active than those of cattle. Thus far, we have not acquired adequate data in other species. An

attempt was made to dissect the cortex and medullary tissue in one set of beef adrenals. It is probable that the cortex samples were free of medulla, but the same cannot be said for the reverse. We cannot, therefore, say with certainty that

Table II

CONCENTRATION OF STEROID 3β-OL DEHYDROGENASE IN TISSUES

Tissue	*Sample*	*αβ-Unsat Ketone formed μ moles/g /3 hr.*
Rat Adrenals	8	72·8 (64·1–88 1)
Mouse Adrenals	3	89 4 (69 1–119)
Beef Adrenals	10	33·4 (23 2–47·6)
Whole Beef Adrenals	3	24 0 (23 2–24 4)
Cortex same adrenal (C)	3	37 1 (36·8–37·2)
Medulla same adrenal (M)	3	15 7 (15 2–16 4)
Activity whole adrenal ~ 3 pts (C)+1 pt (M)		
Normal Rat Testes	20	1·51 (1 11–2·15)
Rat Testes, 3 wk. hypophysectomized	6	1·67 (0·98–2·36)
,, ,, ,, ,, CG inj	3	5 04 (4 04–5 64)
,, ,, ,, ,,	3	5 56 (4 28–6 68)
,, ,, ,, ,, CG inj	5	26·5 (21 0–29 2)
Human Placenta, 10 wk.	1	2 47
Rat Placenta, 16 days	3	0 57 (0 54–0·60)

the medulla contains any enzyme. Certainly the cortical cells contain much more activity.

Normal rat testes have a relatively low concentration because of the dilution of the hormone-forming cells with the mass of spermatogenic tissue. When, however, the testis is allowed to atrophy after hypophysectomy until the

seminiferous epithelium has practically disappeared, and the animal is then injected with chorionic gonadotrophin until the interstitial cells have regenerated, the activity per unit mass is quite high (Table II). Of course, in such preparations there is still a relatively large amount of connective tissue, so that the activity of the interstitial cells themselves must be considerably higher.

Not only do normal hormone-forming cells contain this enzyme, but tumours of endocrine organs which show evidence of forming steroid hormones also contain it. As Table III indicates, there is a considerable variation in the concentration. However, the first series of tumours listed in the

Table III

STEROID 3β-OL DEHYDROGENASE ACTIVITY OF VARIOUS TUMOURS

Tumour	*μmoles Progesterone formed per g tumour*
Mouse adrenal tumour transplant	0 0
,, ,, ,, ,,	5 8
,, ,, ,, ,,	8 5
,, ,, ,, ,,	10 0
,, ,, ,, ,,	17 2
,, ,, ,, ,,	9 3
,, testicular tumour œstrogen produced	4 9
,, ,, ,, ,, ,,	10 2
Human adrenal carcinoma with Cushing's disease	6 3
,, seminoma, no hormonal function	0 0

table was a group of adrenal tumours which had been produced in mice by early castration. These tumours had then been transplanted to normal mice It will be seen that the different transplants (which were each from different parent tumours) varied from no measurable activity to levels which were about 1/4 that of normal rat adrenals. Fig. 2 shows that, with one exception, the size of the seminal vesicles of the recipients was approximately proportional to the enzymic activity of the tumour. The point representing zero activity corresponds both with the size of the seminal vesicles of the

mouse having the tumour with no enzymic activity and with that of the vesicles of the uninoculated controls.

The two samples of human tumours were taken from hepatic metastases. In the case of the seminoma the original tumour had been removed at a previous admission and was not available. In the case with Cushing's disease the original tumour gave values similar to those of the metastatic tissue.

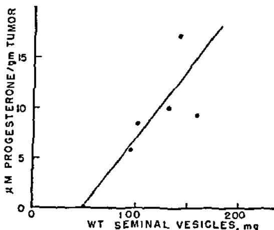


FIG. 2. Relation between the steroid-3β-ol dehydrogenase activity of mouse adrenal carcinomas and their androgenic potency.

The seminoma showed no signs of endocrine activity. The results so far with tumours lead us to the conclusion that there is probably a direct relation between the enzymic activity and the degree of functional differentiation which has been maintained.

The observations on the reduction of the enzyme content in the testes of hypophysectomized rats led us to investigate the relation between the enzyme and gonadotrophins. First, a study of the relation between testicular atrophy, seminal vesicle atrophy, and enzymic activity was carried out. Fig. 3 shows that the disappearance of the enzyme was rapid during the first five days after hypophysectomy. At about

seven days the total activity of enzyme reached a base level which was maintained for the balance of the seven weeks of observation. Since the seminiferous epithelium atrophied more slowly, the concentration of enzyme reached a low point at the 5–7 day period and then slowly increased as the tubular

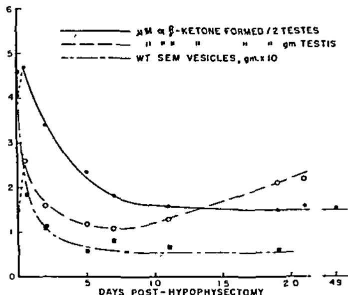


FIG. 3 Effect of hypophysectomy on the steroid-3β-ol dehydrogenase activity of rat testes.

tissue disappeared. The curve of seminal vesicle size followed the general form of the total enzyme activity, but seemed to decrease somewhat more rapidly at first. This could be due either to a certain basic level of hormone needed for significant maintenance, or because the gonadotrophin plays a more important rôle in some other enzyme system involved in synthesis.

The injection of chorionic gonadotrophin produces the same total increase in enzyme activity (Table IV) irrespective of the degree of atrophy of the total testis, provided the

residual activity has reached the base level. There seems to be a close relation, therefore, between the enzyme and the trophic hormone. Whether this is due to synthesis of the specific protein or to activation of the system is an intriguing question. Brady (1951) has reported that the incorporation of isotopic acetate into testosterone *in vitro* was increased tenfold by the introduction of high concentrations of chorionic gonadotrophin into the medium, and Savard, Dorfman and

Table IV

EFFECT OF INJECTED CHORIONIC GONADOTROPHIN ON ENZYMIC ACTIVITY OF HYPOPHYSECTOMIZED RAT TESTES

Substrate	*Pregn-5-en-3β-ol-20-one*		*αβ-Unsat Ketone formed*	
Post Operative Time	*Treatment*	*Ac Wt 2 Testes moles*	*μ moles/g.*	*μ moles/2 Testes*
3 weeks	inj *	0 893	15 9	14 3
	non-inj	0 731	2·14	1 60
7 weeks	inj *	0·520	27 4	14 2
	non-inj	0 250	6 20	1 53

*100 i u Chorionic Gonadotrophin daily for 6 days

Poutasse (1952) have found an increase in incorporation of acetate during perfusion. When, however, rat testis tissue was incubated with chorionic gonadotrophin, either simultaneously with the steroid substrate or before the introduction of the steroid and the DPN, there was no increase in activity. It seems therefore that either our conditions were not suitable or the hormone acts differently on some other enzyme in the synthetic chain.

While we do not have so extensive a study of the disappearance of the enzyme from the adrenals after hypophysectomy, it will be seen in Fig. 4 that a similar reduction occurs after the operation, although the fall is not so great. There also seems to be a tendency for the concentration to increase with time, as though there were a slower atrophy of non-enzymic tissue. The results of injections of pituitary hormones are not yet available.

The presence of relatively large amounts of 3β-hydroxy steroids in the glands of origin, while the 3α-hydroxy compounds predominate in the excretory products, led to the hypothesis that the enzymes involved in synthesis may be sterically specific for the 3β isomer while those involved in

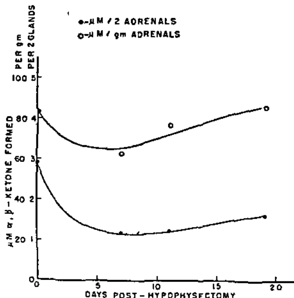


FIG 4 Effect of hypophysectomy on the steroid-3β-ol dehydrogenase activity of rat adrenals

degradation form mainly the 3α structure. We therefore investigated the substrate specificity of the oxidizing enzyme. As seen in Table V the compounds having a 3β-hydroxy group are metabolized much more rapidly than their 3α isomers. The slight amount of oxidation of the latter may be due to the presence of small amounts of the 3β isomers as impurities, since the 3α compounds were prepared from mixtures in which the 3β forms predominated

Other structural characteristics of the steroids are not so crucial. The presence or the absence of the double bond seemed to make little difference in the oxidative rate. Either Δ^5- or Δ^4-unsaturated compounds were metabolized at about the same rates as saturated 3β-alcohols. Androst-5-en-3β-ol was metabolized at a significant rate, but an alcohol or ketone

Table V

EFFECT OF STERIC POSITION ON METABOLISM OF 3-HYDROXY STEROIDS

Steroid	*Tissue*	*μ moles Ketone per g tissue*
Androst-5-en-3β-ol-17-one	Hypox.* Testes CG-Inj	20·0 (10·2–20·8)
Androst-5-ene-3β·17β-diol	” ” ”	25·2 (21 0–26 4)
Androst-5-en-3β-ol	” ” ”	4 4 (4 0–4·8)
Androst-4-ene-3α:17β-diol	Beef Adrenals	1·2 (1·2–1·2)
Androst-4-ene-3β 17β-diol	” ”	27·6 (25·6–29·6)
Pregnane-3α:20α-diol	” ”	0·3?
17α-methylandrost-5-ene-3β 17β-diol	” ”	9·6 (8·8–10·8)
Androstane-3β.17α-diol	Rat Adrenals	81·6 (77·6, 86·0)
Pregn-5-en-3β-ol-20-one	” ”	69·8
Androstane-3β.17α-diol	Normal Rat Testes	1·17
Androstane-3α 17α-diol	” ” ”	0·16
Pregn-5-en-3β-ol-20-one	” ” ”	1·40

*Hypophysectomized

group in position 17 or 20 markedly increased the rate of oxidation. The presence of a methyl group on carbon-17 decreased the rate of reaction. This was probably due to steric hindrance.

The synthesis of the adrenal hormones involves a series of further oxidations at positions 11, 21 and 17. Enzymes for each of these oxidations appear to be present in adrenal homogenates. Sweat (1952) and Hayano and Dorfman (1952) have described the characteristics of the enzyme which catalyses oxidation at position 11. This seems to be the only one

which is not present in the cytoplasm. Hayano and Dorfman have also described the characteristics of the enzyme oxidizing carbon-21. In our own laboratory Mr. Plager was the first to report oxidation of carbon-17 by homogenates. He was able to convert progesterone into deoxycorticosterone, corticosterone and hydrocortisone. Yields on spot density were about 15–25 per cent deoxycorticosterone, 10 per cent corticosterone and 10 per cent hydrocortisone. Using [21-^{14}C] progesterone he was able to get 5–10 per cent conversion to Compound F. The enzyme causing the oxidation at carbon-17 seems to be more unstable than the others since holding overnight in the frozen condition reduces the activity. ATP, DPN or TPN, as well as an acid of the Krebs cycle, appear to be essential for these oxidations. I am sure Dr. Dorfman will discuss this series of oxidations in detail.

Once the adrenal steroid hormones are synthesized and secreted the question of their metabolism becomes an intriguing problem. The compounds in the urine which appear to be specifically associated are the glucuronides of the various compounds formed first by reduction of ring *A* as far as the saturated alcohols, and then of the side chain. Where such changes occur is of interest because the answer has a significant bearing on the peripheral utilization theory of regulation of adrenal steroid secretion. Dr. Nelson and his group have studied this problem, both after ACTH stimulation and after intravenous or oral administration of cortisone and hydrocortisone. The levels of 17-hydroxycorticosteroids in samples of blood drawn simultaneously from the brachial artery and saphenous vein of a patient given a single intravenous injection of ACTH were equivalent within the error of the method No significant difference has been found across limbs with severe cellulitis or in generalized infections. The same is true after injections of the steroids themselves. When dogs were infused with cortisone intravenously, the steroid was removed rapidly from the circulation (Fig. 5) but again there was no significant difference between the levels in the arterial circulation and the renal vein. The kidneys, there-

fore, do not appear to play a major rôle in the removal of these compounds. When, however, comparisons were made between the arterial and the hepatic venous blood there was a marked difference (Fig. 6). The liver removed approximately one-third of the steroid as determined by the Nelson

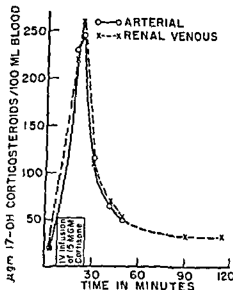


FIG 5. Comparison of levels of 17-hydroxycorticosteroids in blood entering and leaving the kidney

method. As in the case of the other steroids this organ appears to play the key rôle in disposal.

In summary, the pattern by which the adrenal hormones are built up and subsequently disposed of is gradually taking form. The work at Utah has contributed a few facts to the many from other sources which together make a related sequence. One reaction which seems to be very general among the non-benzenoid steroid hormones is the oxidation of 3β-hydroxy steroids to αβ-unsaturated ketones. This system has been studied in some detail. In the adrenal, as distinguished from the corpus luteum and placenta, there are

additional systems which oxidize the 11, 17 and 21 positions. Of these, the only system about which we have contributed significant information is the one oxidizing carbon-17 of the C_{21} compounds to a tertiary alcohol. By using it, isotopic hydrocortisone can be prepared from isotopic progesterone.

The use of the method of Nelson and Samuels for 17-hydroxycorticosteroids in blood or plasma has thrown some

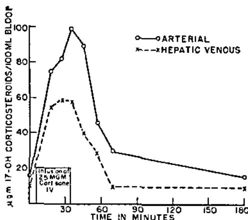


FIG. 6 Comparison of levels of 17-hydroxycorticosteroids in blood entering and leaving the liver.

light on the degradation process. There seems to be little, if any, removal by peripheral tissues even when severely inflamed This makes the peripheral utilization theory in its usual form difficult to maintain.

The liver, on the other hand, removes the free steroids from the blood relatively rapidly. A large portion of the degradative metabolism must take place in this organ. Much remains to be done on the earlier steps in synthesis and on the enzymes involved in the breakdown of the steroids by the liver.

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The Discussion of this paper was postponed until after the paper by Dorfman *et al.*, which follows. (See p. 201).

THE *IN VITRO* SYNTHESIS OF ADRENAL CORTICAL STEROIDS

RALPH I. DORFMAN, MIKA HAYANO, ROBERT HAYNES and KENNETH SAVARD

In a previous Ciba Foundation conference we presented evidence for the 11-hydroxylation of 11-deoxycorticosterone and 17-hydroxy-11-deoxycorticosterone by adrenal tissue slices and homogenates (Dorfman and Hayano, 1952). Since that time, a number of papers have been published on this subject both from our laboratory (Hayano, Dorfman and Prins, 1949; Hayano, Dorfman and Yamada, 1951; Hayano and Dorfman, 1952) and others (McGinty *et al.*, 1950; Savard, Green and Lewis, 1950; Kahnt and Wettstein, 1951; Sweat, 1951; Plager and Samuels, 1952). It has been definitely established that the 11-hydroxylation of deoxycorticosterone and 17-hydroxy-deoxycorticosterone proceeds with the formation of corticosterone and 17-hydroxycorticosterone respectively. This report deals with our recent studies on 11-, 17-, and 21-hydroxylation by adrenal tissue homogenates as well as an *in vitro* action of ACTH on adrenal slices.

Methods

11-, 17- and 21-Hydroxylation Studies

Beef adrenal glands were homogenized in a Waring blendor

steroid. Homogenate residues were prepared using 300 ml. of saline for 100 g. of tissue, strained and centrifuged at 5,000 g for 25 minutes. Subsequent washings of the residue were carried out with the same volume of saline and centrifuged at about 4,000 g for fifteen and ten minutes respectively.

The residue was then taken up in incubation media containing fumarate, magnesium ions and phosphate buffer.

The incubations were carried out in 50 ml. Erlenmeyer flasks at 38° C. for sixty minutes with deoxycorticosterone and 17-hydroxydeoxycorticosterone and for three hours with other steroids. Tissue homogenates in the medium were pipetted into the flasks containing 5 mg. of steroid dissolved in 0·2–0·3 ml. of propylene glycol. The final volume was 7·5 ml. The final concentrations of additions were: sodium fumarate, 0·06M; magnesium ions, 0·006M; and phosphate buffer, 0 02M at *p*H 7·4. The incubate was dialysed against water and the water extract was extracted with chloroform. The chloroform extract was subjected to various further purification steps depending upon the specific aims of the individual experiments.

11-Hydroxylation

Fig. 1 indicates the influence of time of incubation. At the end of ninety minutes all of the deoxycorticosterone (DOC) has been consumed; by far the bulk, about 90 per cent, appears as corticosterone.

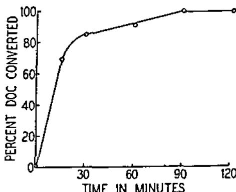


FIG. 1. Percentage of deoxycorticosterone converted with increasing time. Preparation is washed adrenal homogenate residue.

In our earlier experiments we found a pH optimum of 5·9 when deoxycorticosterone glucoside was used as the substrate. When these studies were repeated using the free steroid, a pH optimum of 7·4 was found. The lower pH optimum originally found may well have been the pH optimum of the hydrolysis of the deoxycorticosterone glucoside to the free compound. This is consistent with our finding that no free deoxycorticosterone is found after paper chromatography of the incubate when deoxycorticosterone glucoside is employed as

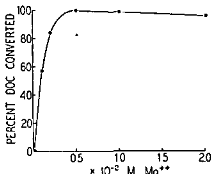


FIG 2. Percentage of deoxycorticosterone converted with increasing concentration of magnesium ions. Preparation is washed adrenal homogenate residue.

the substrate. Apparently the complex is hydrolysed at a relatively slow rate and all the free steroid formed is quickly converted to the 11-hydroxy derivative, that is, corticosterone. If the reaction rate of the deoxycorticosterone glucoside hydrolysis is slow, it would explain the low pH optimum that we reported at an earlier Conference here (Dorfman and Hayano, 1952).

Fig. 2 is concerned with the requirement for magnesium of the 11-hydroxylating enzyme system A concentration of $0 \cdot 6 \times 10^{-2}$M magnesium ions (3 × conc. of DOC) seems to be needed for the system. This is in keeping with our earlier findings.

Fig. 3 is concerned with the equivalent of adrenal tissue that is needed to 11-hydroxylate deoxycorticosterone. We see that a ratio of roughly 700 parts of tissue to one part of

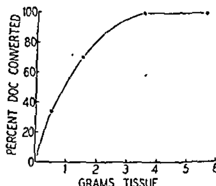


FIG. 3. Percentage of deoxycorticosterone converted with increasing concentrations of washed adrenal homogenate residue.

the steroid is needed for the complete disappearance of deoxycorticosterone during the incubation.

In Fig. 4 it may be seen that in the absence of fumarate the washed adrenal homogenate residue is ineffective in converting deoxycorticosterone to corticosterone. The addition of

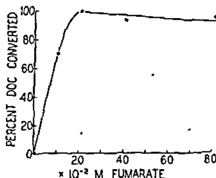


FIG. 4. Percentage of deoxycorticosterone converted with increasing concentration of fumarate. Preparation is washed adrenal homogenate residue.

1×10^{-2}M fumarate ($5 \times$ conc. of DOC) produced a 70 per cent conversion of the steroid, and further doubling the fumarate content produced an optimal conversion.

Table I lists various metabolites that were studied for their ability to substitute for fumarate. Malate and succinate are almost as effective as fumarate. *cis*-Aconitate was able to

Table I

EFFECT OF VARIOUS METABOLITES ON THE SYNTHESIS OF CORTICOSTERONE FROM DEOXYCORTICOSTERONE

Oxidizable Substrate	*Per cent DOC Conversion*
Fumarate 0 06M	100
Malate	80–100
Succinate	75
cis-Aconitate	22
Maleate	20–32?
Citrate	1
α-Ketoglutarate	1
Ascorbate	1
Malonate, acetate, oxaloacetate	0
Lactate, pyruvate, hexose diphosphate	0
Glucose, triose phosphate, aspartate	0

perform the function of fumarate to a limited extent. Fourteen other metabolites listed in Table I were completely inactive.

Deoxycorticosterone acetate as well as the 17-hydroxydeoxycorticosterone acetate can be effectively hydrolysed to the free compounds and 11-hydroxylated by the adrenal homogenate system.

Four C_{19} steroids have been studied. Under conditions similar to those previously employed for DOC, a yield of approximately 30 per cent of androst-4-en-11β-ol-3.17-dione was found when androst-4-ene-3:17-dione was incubated. On incubation of testosterone with the enzyme preparation there is formed a compound which, on the basis of paper mobilities and colour reactions, appears to be androst-4-ene-11β.17β-diol-3-one. Two 6-hydroxy androst-4-ene-3:17-diones have been studied; one the 6α-hydroxy and the other the 6β-hydroxy

compound. In each instance a more polar product has been found by paper chromatography. These products have not been isolated, but again there is a strong likelihood that the 11β-hydroxy derivatives have been formed by the enzyme system.

A variety of C_{21} steroids have been incubated with the washed residue preparation to study the specificity of the 11-hydroxylating enzyme system. These include 5α-pregnan-21-ol-3:20-dione, pregn-5-ene-3β: 21-diol-20-one, progesterone, 17-hydroxyprogesterone, and pregnan-21-ol-3:20-dione. Recently we have demonstrated that 5α-pregnan-21-ol-3 20-dione can be 11-hydroxylated. The 11-hydroxy derivative was isolated from the incubate and identified by m.p. determinations, specific rotation and infra-red analysis of this compound and comparison of the isolated material with an authentic sample which was synthesized from corticosterone by hydrogenation in the presence of palladium black.

Table II lists the relative yields of 11-hydroxy derivatives from various steroids.

17- and 21-Hydroxylation

In a series of experiments, attempts were made to produce selectively 21-hydroxylation using 21-deoxycortisone, progesterone and 17-hydroxyprogesterone as substrates. In each instance we were able to demonstrate by paper chromatography, infra-red analysis, or both, the appearance of the corresponding 21-hydroxy derivative. Specifically, cortisone has been identified after incubation of 21-deoxycortisone, corticosterone from progesterone, and 17-hydroxycorticosterone from 17-hydroxyprogesterone.

The 21-hydroxylating reaction as well as 17-hydroxylation were demonstrated when experiments were done using [4-^{14}C] progesterone and [21-^{14}C] deoxycorticosterone acetate.

In the first experiment involving progesterone, 6 g. of adrenal tissue as the crude homogenate were incubated with 2·464 mg. of ^{14}C-labelled steroid. After addition of carrier steroids, and many fractionations involving both paper and

silica gel chromatography, two pure compounds were isolated. The first was corticosterone, m.p. 184–185° (uncorr.), having a constant specific activity of 4,150 counts/mg./min. and homogeneous by infra-red analysis. This indicated a 64 per cent conversion of progesterone to corticosterone. This definitely

Table II

RELATIVE YIELDS OF 11-HYDROXY-DERIVATIVES FROM VARIOUS STEROIDS (5 G. EQUIVALENT OF WASHED ADRENAL HOMOGENATE RESIDUE AND 5 MG. OF STEROID)

Substrate	*Product*	*Per cent Yield*
Deoxycorticosterone	Corticosterone	90–95
Deoxycorticosterone acetate	Corticosterone	90–95
Deoxycorticosterone glucoside	Corticosterone	30
17-Hydroxydeoxycorticosterone	17-Hydroxycorticosterone	90–95
17-Hydroxydeoxycorticosterone acetate	17-Hydroxycorticosterone	90–95
5α-Pregnan-21-ol-3 20-dione	5α-Pregnane-11β 21-diol-3 20-dione	30
Pregnan-21-ol-3 20-dione acetate	Pregnane-11β 21-diol-3 20-dione	20
	Pregnan-21-ol-3 20-dione	80
Androst-4-ene-3 17-dione	Androst-4-en-11β-ol-3 17-dione	25
Pregn-5-ene-3β 21-diol-20-one	Pregn-5-ene-3β 11β:21-triol-20-one ?	15
Testosterone	Androst-4-ene-11β 17β-diol-3-one ?	10
Pregn-5-en-3β-ol-20-one	No product	
Progesterone	No product	
17β-Hydroxyprogesterone	No product	
Androsterone	No product	

indicated the 21-hydroxylation as well as the 11-hydroxylation. A 1 per cent yield of 17-hydroxycorticosterone was calculated when 17-hydroxycorticosterone, m.p. 197—204° (uncorr.), was isolated, having specific activity of 1,950 counts/mg./min.

In the second experiment, 2·40 mg. of [4-^{14}C] deoxycorticosterone was incubated with 6 g. of adrenal tissue homogenate. A total of 5·7 mg. of carrier 17-hydroxycorticosterone and 13·6 of carrier corticosterone was added. Again, by the

use of paper chromatographic techniques and silica gel chromatography, two fractions were isolated. One proved to be pure corticosterone, m.p. 181–184° (uncorr.), which had a specific activity of $1 \cdot 5 \times 10^5$ counts/mg./min. The second compound which indicated that the 17-hydroxylation actually occurred was 17-hydroxycorticosterone, m.p. 200-204° (uncorr.) with a specific activity of 17,670 counts/mg./min, amounting to a 2 per cent yield from the original deoxycorticosterone.

The washed residue preparation was found to be inactive with respect to 21-hydroxylation although the 11-hydroxylating potency has repeatedly been of a high order. This would appear to make it quite likely that the 11-hydroxylating and 21-hydroxylating enzyme systems are discrete entities. Further work along this line is in progress.

In Vitro Action of ACTH

Recently, by two separate methods, a stimulatory effect of added ACTH on corticosteroid hormone production by adrenal cortical slices under conventional incubation conditions has been demonstrated. Since the completion of the first phase of this work, Saffran, Grad and Bayliss (1952) have reported that the addition of ACTH to rat adrenal halves *in vitro* results in an increased output of adrenal cortical hormones as measured by the U.V. absorption at 240 mμ of chloroform-soluble substances and by a biological assay method involving the eosinophil drop of the adrenalectomized mouse. Our experiments have involved the incubation of beef adrenal slices in the presence of [^{14}C] sodium acetate. With such a system we have been able to show that the 17-hydroxycorticosterone formed during a two-hour incubation at 37° C. in an atmosphere of 95 per cent O_2 and 5 per cent CO_2 contained a greater specific activity when ACTH was present. The ^{14}C-labelled ... corticosterone formed during these incubations has

the adrenosterone was found to retain the same order of specific

radioactivity as found for the 17-hydroxycorticosteroids. The results of these experiments show that incorporation of ^{14}C into 17-hydroxycorticosterone was increased twofold in the presence of ACTH (Table III). In a second series of experiments, it was demonstrated that added ACTH increased the

Table III

INFLUENCE OF ACTH ON *in vitro* INCORPORATION OF [1-^{14}C] ACETATE INTO 17-HYDROXYCORTICOSTERONE

Acetate μc./g tissue	Compound F			
	Control		Added ACTH	
	Amount of carrier F added (mg)	Specific activity of isolated F counts/mg/min.	Amount of carrier F added (mg)	Specific activity of isolated F counts/mg/min
13 5	0 5	46	0 5	92
83	2 0	102,000	2 0	200,000
94	2 15	3,370	2 15	7,350

output of formaldehydogenic steroids from adrenal slices. The corticoids produced by incubation were chromatographed on silica gel after preliminary extraction and the amounts of corticoids present were estimated by periodate oxidation and the appropriate column fractions followed by determination of the liberated formaldehyde. Data from one such experiment are given in Table IV.

Summary

We have presented evidence indicating the presence in adrenal tissue of enzyme systems capable of carrying out 11-hydroxylation, 17-hydroxylation and 21-hydroxylation in certain steroids. Various characteristics of the 11-hydroxylating enzyme have been indicated and it has been shown that the enzyme system can produce its reaction on a variety of C_{19} and C_{21} steroid substrates. Finally, an *in vitro* system has been described in which ACTH has been shown to have a direct effect upon adrenal slices This effect appears to be one of stimulating the production of adrenal cortical steroids.

Table IV

INFLUENCE OF ACTH ON *in vitro* OUTPUT OF FORMALDEHYDOGENIC SUBSTANCES (VALUE IN μG. FORMALDEHYDOGENIC STEROID PER GRAM OF TISSUE)

Steroid initially present	Steroid at end of incubation						Output			
	Control			ACTH			Control		ACTH	
	No of vessels	Av	Range	No of vessels	Av	Range	Av	Range	Av.	Range
44	4	71	62–78	4	126	107–148	27	18–34	82	63–104

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DISCUSSION

MARRIAN I wasn't quite clear, Dr. Dorfman, about your statement that with the 11-hydroxylating enzyme there was no recognizable product produced from progesterone. Did you mean there was no recognizable α-ketolic product?

DORFMAN· Yes.

TAIT. As our work in regard to the *A* ring structure of our potent mineralocorticoid has been mentioned, I should like to take this opportunity to clarify this point. The first figure shows the position of the

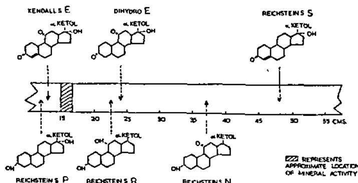


FIG 1 Position of mineral activity on typical 3-day chromatogram compared with other adrenal steroids.

from cortisone when the paper is tested by the soda-fluorescence method of Bush, which we have confirmed is highly specific for Δ^4-unsaturated 3-ketones. However, the possibility that such evidence could be masked by other compounds is quite real. The compound is probably present in very small amounts and there are undoubtedly a large number of other compounds in this region, e.g., there is a phenol which runs just ahead of the activity. The second figure shows the evidence for the formation of a polyacetate derivative on treatment of the active compound with pyridine and acetic anhydride. The acetyl derivative

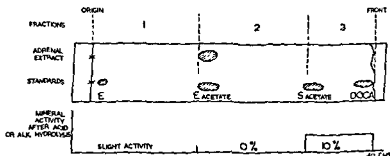


FIG. 2. Typical appearance of a 3-hour chromatogram on benzene-formamide system, showing position of mineral activity after hydrolysis of acetyl derivative of the mineralocorticoid.

... be seen and by hydrolysis, as much as

We feel that conclusions regarding structure drawn *from* work on ultramicro quantities are tentative only, and further work must be done on milligram quantities before the *A* ring structure is established.

DORFMAN: I would like to raise a question about the 11β-hydroxyl- ... and medullary tissue. ... have studied this point. ... arated the cortical tissue ... nificant amount of 11β-hydroxylating enzyme in the medullary tissue.

KAHNT: I would like to mention the results of some unpublished work in our laboratories. We tried to find out whether the adrenal cortex or the adrenal medulla is responsible for the 11-hydroxylation We separated fresh bovine adrenals into two parts, one consisting

content of the medulla did not have any effect on the yield of Compound F. We conclude that the adrenal medulla does not contain any appreciable amount of the 11-hydroxylating enzyme, and that neither the medulla nor pure adrenaline have any specific action on the *in vitro* production of Compound F.

BUSH: *It is difficult to separate the adrenal cortex from the medulla,*

luteal phase or in pregnancy, can go right to the centre of the medulla.

KAHNT: I would
ACTH on the proc
that the addition
amount of 11-hyd
replace the essential fumaric acid in our experiments, but ACTH alone does not show any activity. Isn't it possible that the ACTH forms a certain amount of ascorbic acid or ascorbic acid-like substances in your homogenate?

DORFMAN I believe you used a crude homogenate for your studies, while we used a washed residue. The difference is due to the type of enzyme preparation employed.

HECHTER: Dr. Dorfman, would you care to comment on possible mechanisms of the various oxidations? With a houseful of experts on organic reactions, perhaps they could throw some light on what's going on.

DORFMAN: From a biochemical point of view it's very tempting to

been done yet.

DJERASSI: It is interesting to notice that all the hydroxylated

hormones formed by either perfusion or enzymic incubation are invariably 11β-hydroxy compounds, while the compounds formed by microbiological oxidation can be both β and α. The more exciting one is the 11α-hydroxy compound, because that raises the question of whether it has any biological functions. To a chemist the 11α compound is much more attractive mechanistically speaking, and it is the one that you would expect on purely steric grounds Why we have thus rather marked differentiation I do not know, but it is quite possible that there might be two entirely different mechanisms: one a hydration mechanism, going through some dehydrogenase system and then addition of water; the other one through something else, possibly reduction of an initially formed ketone. I think that has been excluded in the perfusion mechanism because if cortisone is perfused one does not get F On the other hand, Dr Zaffaroni and Dr. Rubin at Syntex

SONDHEIMER If this were to be pictured as dehydrogenation, followed by any simple type of hydration, one would expect the 11α-hydroxy compound to be formed rather than the 11β.

DJERASSI· Yes, certainly.

HECHTER. I would like to mention some interesting findings that have come out of our adrenal perfusion data. If C-21 is hydroxylated, the C-17 hydroxyl function is difficult to introduce. Thus, if corticosterone or deoxycorticosterone is perfused, little or no 17-hydroxycorticosterone is formed. Dr. Dorfman has just indicated, using a radioactive tracer technique, that this reaction does proceed in homogenates but to a very limited extent. Why is progesterone, but not DOC, readily hydroxylated at C-17?

Finally, I wish to raise a few points about the 11β hydroxy mechanism. Dr. Harold Levy at the Worcester Foundation, just before

another would replace

configuration. Where might these postulated hydroxyl free radicals come from? The C-11 hydroxylation appears to require dicarboxylic acid, whether malate or fumarate. One might assume that the dicarboxylic acid is oxidized through the various hydrogen transport systems, ultimately passing through a hydrogen peroxide stage. The peroxide formed might then be the precursor of the hydroxyl free radicals which he regards as the driving force for the 11β-hydroxylation reaction.

REICHSTEIN: I don't think it's any good to speculate much about this until Dr. Dorfman has made his crucial experiment, whether the 9(11)-unsaturated compound does add a molecule of water, because that would be the easiest explanation. If you first add a proton on the 9-position, then you would have *trans* addition of water and the 11 would be β, because the 9 would be α. I think the crucial experiment is really to see whether there is an unsaturated intermediate, and I would be very much astonished if there were not.

[illegible]

possibly involving a *cis* addition

DJERASSI: Or no hydration mechanism at all

[illegible]

desired.

REICHSTEIN: The reason for such ideas is that in the well known

[illegible]

SHOPPEE: The hydroxyl radical is not a charged body, as opposed to an ion, so that the usual stereochemical considerations, which after all are fundamentally electrical in character, will not operate to anything like the same extent.

HEMS. Somebody recently isolated
microbiolo,
in hydroxy
peculiar.
neither hy
oxidation of the enol I suggest Dr. Dorfman might add to his list the compound with a double bond at 8(9).

HECHTER. In the case of the 11-hydroxylation system in the adrenal, any mechanism which purports to explain this reaction must include, in addition to the active protein, an explanation of the rôle of magnesium, and of the dicarboxylic acid. The latter factors must be considered as important as some of the steric factors and intermediates which have been mentioned.

SAMUELS: I would like to support what Dr. Hechter has just said, that we mustn't forget that this is happening on the surface of a protein molecule, not in solution. As Dr Vennesland has so well demonstrated,

DORFMAN. I might say that we haven't found a substitute for magnesium. Manganese, for example, is inactive.

BARTON: I would like to ask one possibly rather foolish question,

after incubation of ^{14}C labelled progesterone, I think it's unlikely.

BARTON: You had this labelled in ring *A*?

DORFMAN: Yes

REICHSTEIN: Dr. Samuels, you said that the enzyme system which converts pregnenolone into progesterone finishes up with 75 per cent equilibrium. Is that a true equilibrium? Can you do the reverse reaction, so that progesterone yields 25 per cent pregnenolone?

SAMUELS: That experiment was carried out just before I left and I don't have the final results yet. I can say that there was reduction, but whether we get the same equilibrium I don't know.

I would like to ask Dr. Dorfman about the conditions he used for the radioactive Compound F results Were those one-hour incubations?

DORFMAN They were 2-hour incubations using a ratio of 3,000 1 tissue to steroid, at *p*H 7 1 and phosphate buffer.

SAMUELS: Mr. Plager was able to get Compound F formed from

sequence. Also he found a bicarbonate buffer system seemed to work

ration?

DORFMAN Probably some

FOLLEY. And did you make any measurements of oxygen uptake, without and with ACTH?

DORFMAN: With the homogenate we have—it was a low oxygen uptake. With the residue, I don't recall that we have.

FOLLEY· Did you get an increase in the oxygen uptake with the fumarate, as against no fumarate?

DORFMAN. Practically none

SAMUELS· I would have anticipated increased oxidation, but it would be difficult to relate it to the oxidation of steroids, since the mitochondria have the enzymes for oxidation via the Krebs cycle In regard to this, Mr. Plager has prepared highly ultracentrifuged fractions that are free from mitochondria, and oxidations at carbons 21 and 17 both seem to occur Of course the 11-hydroxylation disappears from these. The 17- oxidase seems to be much more unstable. We've used relatively fresh glands

HECHTER Dr. Samuels, what concentrations of ATP were used in the experiments from your laboratory relative to those employed by Dr. Dorfman?

SAMUELS I can't say.

DORFMAN. Our experiments with ATP and DPN have been indirect.

DORFMAN· Yes

SAMUELS. Perhaps the DPN is involved in rebuilding ATP. Undoubtedly there are ATPases present

DORFMAN· In another indirect experiment we incubated deoxycorticosterone with our residue, centrifuged, removed the steroid, and then repeated that process 3 or 4 times, and we eventually got to the place where only a trace of corticosterone is produced At this point addition of ATP and DPN seems to bring the enzymic activity back.

pound F depending on the amount of oxygen Have you done any work on this point?

DORFMAN We have done experiments in air and in 95 per cent oxygen, and we did not find a real difference. We have not studied the factor of oxygen further.

I would like to bring up the question of 11-hydroxylation by liver

KAHNT: By paper chromatography, including runs with mixtures of the fraction and pure Compound F.

DORFMAN: We have thus far been unable to demonstrate 11β-hydroxylation in liver or kidney tissue.

DJERASSI. Dr. Zaffaroni tried to repeat Dr. Kahnt's experiment under the same conditions as he published in *Helvetica*, and he got no F at all

KAHNT: With beef liver?

DJERASSI. I think with rabbit liver.

HECHTER: In connection with Dr. Kahnt's characterization of the

deoxycorticosterone we found that many ketol metabolites formed from deoxycorticosterone give this blue coloration with iodine. One of these compounds had a polarity practically identical with cortisone. When, however, enough of this material was obtained, infra-red analysis conclusively revealed that this substance was not cortisone. The reason I've mentioned this in detail is to illustrate the possibility that characterization of a substance by paper chromatography may be completely misleading, unless one does more than simple paper chromatography.

SONDHEIMER: Would you consider that a careful mixed paper chromatogram is more justified than a single chromatogram?

guide to homogeneity. It must be remembered that you collect fractions by solvent partition, and the actual systems available for chromatography, particularly of these steroids, are all very similar in their polarity, so that on general grounds you wouldn't expect the partition coefficients to be very different. There are only really about two or [illegible]

MARRIAN. I've been waiting a long time for somebody to say this!

BUSH: I think Dr. Morris said something which is certainly true, but perhaps conveys a rather one-sided impression. I know also that our Chairman is very keen—I think rightly so—on the isolation of materials in biochemical experiments and characterization by classical means. But it does seem to me that in the field of steroids neither method is ideal, if you can use both you're pretty sure, but if you use only one there's very little to choose between classical methods or chromatography. The surest way is to do, as Dr Zaffaroni has done, a series of reactions giving various derivatives and show that all the derivatives behave identically But I may say that my experience of people who hand me samples to chromatograph "just to check up that it is identical with X or Y", or that "it is pure", comes out very much in favour of chromatography I've known substances which were apparently identical by three classical tests on three derivatives and two classical tests of the esters of the derivatives, and after chromatographing the products I've had to tell the astonished chemist that in fact neither compound was homogeneous and only one perhaps of three compounds in each was identical in the two mixtures After four or five weeks' hard work, in which his classical methods had been applied rather more rigorously, he's been able to confirm my verdict One more example may be quoted to demonstrate this I examined three commercial samples of Reich- [illegible]

I would like to bring up the question of 11-hydroxylation by liver and kidney tissues which Dr. Kahnt reported recently.

KAHNT: We obtained a small amount of Compound F in experiments with rabbit and beef liver. The conversion of Substance S to F amounted to only 1–5 per cent. In the liver homogenate without added Substance S we did not detect any Compound F. The same holds for kidney.

HECHTER: Would you tell us how you characterized the F?

KAHNT: By paper chromatography, including runs with mixtures of the fraction and pure Compound F.

SAMUELS. Did you repeat it after forming the acetate?

KAHNT: No. But the crude extract has been chromatographed twice on silica gel. We shall check the fraction again, but I am convinced that I got Compound F.

DORFMAN: We have thus far been unable to demonstrate 11β-hydroxylation in liver or kidney tissue.

DJERASSI: Dr. Zaffaroni tried to repeat Dr. Kahnt's experiment under the same conditions as he published in *Helvetica*, and he got no F at all.

KAHNT: With beef liver?

DJERASSI I think with rabbit liver.

HECHTER In connection with Dr. Kahnt's characterization of the "F" arising from liver incubation, I should like to relate some of our experiences with DOC perfused through liver. Seneca and co-workers (*Science*, 1950, **112**, 524) reported that deoxycorticosterone incubated with a variety of tissues, including liver, gave rise to cortisone, which they identified by paper chromatography The material had polarity similar to cortisone, it was a ketol, and it gave a blue spot with the iodine reagent described by Zaffaroni. In our liver perfusion studies with deoxycorticosterone we found that many ketol metabolites formed from deoxycorticosterone give this blue coloration with iodine. One of these compounds had a polarity practically identical with cortisone When, however, enough of this material was obtained, infra-red analysis conclusively revealed that this substance was not cortisone. The reason I've mentioned this in detail is to illustrate the possibility that characterization of a substance by paper chromatography may be completely misleading, unless one does more than simple paper chromatography.

SONDHEIMER: Would you consider that a careful mixed paper chromatogram is more justified than a single chromatogram?

[illegible] However

[illegible] it is useful to have some nice crystalline material as well, to

[illegible]

been attracted by some form of "unitarian" hypothesis of adrenal secretion (see review by Sayers, 1950).

The purpose of this paper is to try and decide just how far the available evidence from direct studies of adrenal venous

Table I
THEORIES OF ADRENAL CORTICAL FUNCTION

Theory	*Postulated secretion products*	*Main sources of evidence*
"N and S" theory (Albright)	"S" hormone such as Kendall's Compound F "N" hormone, probably a 17-ketosteroid	Pathology of adrenal hyperfunction (man)
"Dual theory" 1 (Greep)	"Sugar hormone" from Z fasciculata, "Salt hormone" from Z glomerulosa.	Histological changes induced by hormone administration and/or changes in diet (rats)
"Dual theory" 2 (Selye)	Glucocorticoids and mineralocorticoids, as antagonists in pathological processes	Effects of DCA administration Effects of "stress", formalin "arthritis", etc., etc, etc. (rats)
"Unitarian theory" (Conn)	Compound F secreted, acting both as sugar and salt hormone Androgenic hormones produced by extra-adrenal conversion of Compound F.	Effects of Compounds F and E, and of ACTH, in man.
Totipotent theory 1 (Kendall)	A mixture similar to gland extracts is secreted No single hormone is adequate to account for adrenal function	Complexity of syndrome caused by adrenalectomy. Effects of adrenal steroids and extracts on adrenalectomized animals (rats, dogs, cats)
Totipotent theory 2 ("Embryological theory")	Derived embryologically from the interrenal body, the adrenal is held to be relatively undifferentiated and to secrete all types of steroid hormone.	Embryology and histology of adrenals and gonads Adrenal extracts contain progesterone, adrenosterone, and œstrone Sex differences in adrenal. Adrenogenital syndrome, etc, etc

SPECIES DIFFERENCES AND OTHER FACTORS INFLUENCING ADRENOCORTICAL SECRETION

I. E. BUSH

The biochemistry of the synthesis of steroids by the adrenal cortex has been thoroughly discussed by previous speakers so that this paper will deal mainly with physiological aspects of adrenocortical secretion and will be based mainly on experiments in which adrenal venous blood has been collected *in vivo* and examined chemically.

The chemical study of adrenal venous blood in recent years has been stimulated principally by the desire to discover the chemical basis of adrenocortical function. It is worth noting that both this particular study and the idea behind it originated some time ago, for Virchow in 1857 noted that blood from the adrenal vein gave the same colour reaction with ferric chloride (Vulpian's reaction) that was given by the adrenal medulla, thus providing the first direct evidence in support of the theory of the "blood secreting" function of the endocrine organs (de Bordeu, 1775). Vogt's work (1943) gave the first indication that chemical studies on the secretion of the adrenal cortex should be feasible and profitable.

Six more or less distinct theories of the nature of the hormone, or hormones, secreted by the adrenal cortex and their physiological roles are summarized in Table I. It will be noted that all agree in postulating the secretion of a "sugar" or "carbohydrate regulating" hormone, and that all are based mainly on indirect methods of investigation. Otherwise they are rather different and it is not an entirely academic task to try and decide which of them, if any, is nearest to the truth. Recently it has been emphasized that few steroid hormones have entirely limited and specific pharmacological actions and for this and other reasons many endocrinologists have

(*f*) Reflex photography in ultraviolet light ($\lambda = 253 \cdot 7$ mμ.);

(*g*) Colour reaction with iodine in light petroleum.

After these experiments further work on each species was carried out in which the R_F value of the free compounds and their reaction with NaOH were used for characterization and semiquantitative estimation. Known amounts of at least two reference substances were run on the same chromatogram in parallel with the blood extracts. This simplified routine analytical technique was shown by work on pure steroids and gland extracts to be reliable and capable of distinguishing all six of the known active cortical steroids, three other $\alpha\beta$-unsaturated 3-ketosteroids in gland extracts, and, in conjunction with triphenyltetrazolium chloride, three other known (inactive) α-ketols of gland extracts.

Estimation of quantities was only approximate (visual comparison of spots with reference substances) and had an error of ± 10 to ± 40 per cent over the range of quantity usually found in the extracts. The limit of detection of $\alpha\beta$-unsaturated 3-ketosteroids was $0 \cdot 25$–$1 \cdot 0$ μg. per spot using NaOH, and $2 \cdot 0$–$5 \cdot 0$ μg. per spot of reducing steroids with triphenyltetrazolium chloride. Recovery values for the extraction of 1–30 μg. amounts of corticosterone, 11-dehydro-17-hydroxycorticosterone, and 17-hydroxycorticosterone from blood by the method used was 85–105 per cent.

Perfusion experiments were carried out by a technique similar to that of Vogt (1951) and involved no hæmostasis during the transfer of the gland to the perfusion circuit.

Results

1. Secretion of the Adrenal Cortex in the Eviscerated Animal with Splanchnic Nerves Intact

In all the animals the adrenal venous blood contained 17-hydroxycorticosterone and/or corticosterone. Singly or together these two compounds made up 85–100 per cent of the total quantity of observable $\alpha\beta$-unsaturated 3-ketosteroids in

blood collected *in vivo* helps us to understand the physiological functions of the adrenal cortex. It will be found that the major contribution of such studies to date has been to provide evidence of a critical type: that is to say, evidence which enables us to decide the merits of existing theories of adrenal cortical function rather than evidence which provides any entirely novel conceptions.

General Methods and Conditions of Experiments

Adrenal venous blood has been collected from anæsthetized, eviscerated animals using techniques similar to those of Vogt (1943). The splanchnic nerves were usually left intact. The figures given for secretion rates are thus probably much larger than occur in undisturbed animals and the physiological conditions of the animals in these experiments could be described as varying from moderate to severe "stress".

The following species have been examined. dog, cat, ferret, rabbits, sheep (with Dr. K. Ferguson at the University Department of Animal Pathology, Cambridge), rat and monkey.

From 4–12 blood samples were drawn over periods ranging from one-and-a-half to six hours and extracts made with ethyl acetate according to a method described at an earlier Ciba Foundation colloquium (Bush, 1953). The purified extracts were examined by paper chromatography (Bush, 1952). Only the essential features of this method will be described here.

Except in the case of the ferret, identification of the main components of the extract was first established for each species by chromatography of several extracts and a variety of methods of examining the chromatograms. Identification was based on:—

(*a*) Fluorescence reaction of free compound with NaOH ($\alpha\beta$-unsaturated 3-ketosteroids);

(*b*) R_F value of free compounds;

(*c*) R_F value of acetates of compounds;

(*d*) Reaction with alkaline $AgNO_3$ or triphenyltetrazolium chloride (TPTZ) (Zaffaroni and Burton, 1951) (α-ketols);

(*e*) Reaction with dinitrophenylhydrazine in 2N-HCl;

Table III

OTHER COMPOUNDS FOUND IN ADRENAL VENOUS BLOOD

Species	*Compounds found*	
Cat	11-dehydro-17-hydroxycorticosterone 11-dehydrocorticosterone	Regularly, up to 13 per cent each of total secretion
	17-hydroxyprogesterone (?)	Very occasionally, up to 4 per cent of total
	11-hydroxyandrost-4-ene-3 17-dione(?)	Very occasionally, up to 2 per cent of total
	"Ai" and "Aii"	Occasionally, up to 3 per cent each of total
Dog	11-dehydro-17-hydroxycorticosterone	Occasionally, up to 0 5 per cent of total
	11-dehydrocorticosterone	Occasionally, up to 0 2 per cent of total
	"Ai"	Occasionally, up to 3 per cent of total
	"Aii"	Occasionally, up to 3 per cent of total
Monkey	11-dehydro-17-hydroxycorticosterone	Occasionally, up to 1 7 per cent of total
	Corticosterone	Occasionally; up to 1 7 per cent of total
Rat	11-hydroxyandrost-4-ene-3 17-dione (?)	Occasionally, up to 40 per cent of total
Sheep	11-dehydro-17-hydroxycorticosterone	Regularly, up to 3 per cent of total.
	11-hydroxyandrost-4-ene-3 17-dione (?)	Regularly, up to 5 per cent of total
	"Aii"	Occasionally; up to 5 per cent of total
	Corticosterone	Occasionally, up to 5 per cent of total

2. Factors Influencing Total Rate of Secretion

(*a*) *Pituitary Gland and Adrenocorticotrophic Hormone (ACTH)*

In the smaller animals it was usually impossible to obtain any increase in secretion rate by giving ACTH (Armour) in

all species except cats, in which they made up only 74–90 per cent of the total. Except in dogs and some cats the secretion rate was large throughout the experiments, and in all animals the rate was large during the first half hour after completing the surgical preparation. Except in two cats the relative proportions of both the major and the minor components of the secretion remained the same throughout each experiment. In the majority of experiments it could be shown from the sensitivity limit of the fluorescence reaction that less than 2·5 per cent of any other single αβ-unsaturated 3-ketone was present in the extracts in terms of the total quantity of 17-hydroxycorticosterone and corticosterone present. These results are summarized in Table II.

Table II

Ratio of Major Steroids in the Adrenal Effluent Blood of Various Species

	Ratio $\frac{\text{Compound F}}{\text{Compound B}}$	*Maximum secretion rates mg./kg. per animal per 24 hr.*
Monkey	>20	3·5–5·5
Dog	6	3·0–8·0
Cat	4–6	3·5–8·5
Sheep	15–20	
Ox	1	(2–3·5)
Ferret	1·5	1·5–4·5
Rabbit	<0·05	1·5–3·5
Rat	<0·05	8–24

Other steroids found less regularly or in much smaller amounts in adrenal venous blood are given in Table III. It is seen that except in the cat and the sheep only traces of 11-ketones were found. The other compounds have not yet been identified completely and their sporadic occurrence has prevented satisfactory examination of their properties. "Ai" and "Aii" are compounds very similar to those found in gland extracts (Bush, 1952).

Table III

OTHER COMPOUNDS FOUND IN ADRENAL VENOUS BLOOD

Species	Compounds found	
Cat	11-dehydro-17-hydroxycorticosterone 11-dehydrocorticosterone	Regularly, up to 13 per cent each of total secretion
	17-hydroxyprogesterone (?)	Very occasionally, up to 4 per cent of total
	11-hydroxyandrost-4-ene-3 17-dione(?)	Very occasionally, up to 2 per cent of total
	"Ai" and "Aii"	Occasionally, up to 3 per cent each of total
Dog	11-dehydro-17-hydroxycorticosterone	Occasionally, up to 0 5 per cent of total.
	11-dehydrocorticosterone	Occasionally, up to 0 2 per cent of total.
	"Ai"	Occasionally, up to 3 per cent of total
	"Aii"	Occasionally, up to 3 per cent of total
Monkey	11-dehydro-17-hydroxycorticosterone	Occasionally, up to 1 7 per cent of total
	Corticosterone	Occasionally; up to 1 7 per cent of total
Rat	11-hydroxyandrost-4-ene-3 17-dione (?)	Occasionally, up to 40 per cent of total
Sheep	11-dehydro-17-hydroxycorticosterone	Regularly, up to 3 per cent of total.
	11-hydroxyandrost-4-ene-3 17-dione (?)	Regularly, up to 5 per cent of total
	"Aii"	Occasionally, up to 5 per cent of total
	Corticosterone	Occasionally, up to 5 per cent of total

2. Factors Influencing Total Rate of Secretion

(a) *Pituitary Gland and Adrenocorticotrophic Hormone (ACTH)*

In the smaller animals it was usually impossible to obtain any increase in secretion rate by giving ACTH (Armour) in

large doses (2–4 i.u./kg./hour) by intravenous infusion or by quick infusions of 1–2 i.u./kg. over 1–5 minutes. The blood loss caused by the collection of samples and the operation involved seemed to cause a prolonged period of maximal rate of secretion which could not be further increased by exogenous ACTH.

In the cat, dog, and sheep, ACTH given from 1–2 hours after completing the preparation caused rapid increases (2- to 3-fold) in secretion rate. In the dog the increase was apparent within 2 minutes, and the peak rate reached within 5–10 minutes of beginning the infusion of ACTH.

One hour after the hemidecapitation (Parkes and Fee, 1929) and hypophysectomy of three rabbits the secretion rate was 1/5–1/10 that usually observed. On giving ACTH intravenously the secretion rate rose rapidly to the normal (maximal) rate found with the pituitary intact. Isolated cat and ferret adrenals perfused with blood drawn from a decapitated donor animal behaved in a similar way. The high *in vivo* rate of secretion persisted for 10–30 minutes and then fell off to 1/5–1/10 the *in vivo* rate within 15–40 minutes. ACTH injected close to the arterial perfusion cannula produced detectable increases in secretion rate within 2–10 minutes.

The lower limit to the latency of action of ACTH was not discovered since it was always smaller than the sampling time necessary for obtaining detectable quantities of steroids (cf., however, Vogt, 1951).

(*b*) *Splanchnic Nerves*

It was found that in cats and dogs which had had both splanchnic nerves cut early in the operation, both the initial secretion rate and the secretion rate an hour later were uniformly lower than in animals with splanchnic nerves intact. This finding needs confirmation on a larger series of animals, but if confirmed it indicates that the rôle of the adrenal medulla in stimulating ACTH release is an important one in this type of "stress", in agreement with the results of Fortier (1951) on the rat.

(c) Blood Flow

It was observed on two occasions that accidental interruptions of blood flow of from 30–60 seconds caused complete cessation of secretion for an observed period of one-and-a-half hours after the accident, although the blood flow was apparently normal throughout the period after its re-establishment.

It was observed in two *in vivo* experiments, and in many early perfusion experiments where vasoconstriction was a difficult technical problem, that when the adrenal blood flow fell to the point where the effluent blood became appreciably "venous" in appearance (adrenal venous blood is normally "arterial" in appearance) the total secretion rate fell to very low levels although good responses to ACTH could still be obtained.

(d) Individual Variation

Animals were drawn at random from laboratory stock and showed considerable variation in the observed "maximal" (unaltered by large doses of ACTH) adrenal secretion rates For any species the largest individual rates observed were from 2–3 times the lowest (see Table II).

(e) Species Variation

The only significant finding as yet has been the very high rate of secretion per unit body-weight of the rat compared with other species (Table II). Other apparent species differences probably depend very greatly on diet and living conditions.

3. Factors Influencing the Composition of the Adrenal Secretion

The ratio 17-hydroxycorticosterone/corticosterone appeared to be a characteristic of each species and, except in the cat, was never observed to change appreciably in the course of any experiment. Spontaneous changes in total secretion rate or those induced by ACTH infusions or other experimental procedures always involved all components of the secretion.

In the cat, however, both these general rules were broken and the ratio 17-hydroxycorticosterone/corticosterone was usually between 7 and 4 but occasionally was as low as 1 or as high as 12·5. Again in two cats (of 14) significant changes in this ratio were caused by injecting large doses of ACTH (see Table IV).

Table IV

An Example of Alteration of the Ratio 17-Hydroxycorticosterone/Corticosterone in the Cat

5 kg ♂, chloralose anæsthesia, not eviscerated.
3 i.u. ACTH (Armour) injected intravenously and
7 i u. ACTH subcutaneously at 15 48 within 10 seconds.
5 min samples from left adrenal. Splanchnic nerves intact.

Time of beginning sample collection	*Secretion rate in μg /min*		*Ratio 17-Hydroxycorticosterone/ Corticosterone*
	17-Hydroxycorticosterone	*Corticosterone*	
11 50	6 0	0 8	7 5
11 55	6 0	0 8	7 5
12 25	6 0	0 4	15 0
15 30	5 0	0 4	12 5
15·41	6 0	0 6	10 0
15 47(ACTH)	8 0	1 6	5 0
15 52	7 0	2 0	3 5
15 57	7 0	3 0	2 35

The only other exceptional case was the rat. Here individuals varied in the ratio of corticosterone to the compound tentatively identified as 11-hydroxyandrost-4-ene-3:17-dione from 80:1 to 3.2. However, the ratio did not change in any individual with changes in total secretion rate.

Discussion

Comparison with other Chemical Studies of Adrenal Venous Blood

These results are in agreement with previous *in vivo* work on the dog by Reich, Nelson and Zaffaroni (1950). There are two major differences, however, when they are compared with

the perfusion experiments of Hechter, Zaffaroni, Jacobsen, Levy, Jeanloz, Schenker and Pincus (1951). In the first place, a very high proportion of the total α-ketols secreted by their isolated beef adrenals was made up of 9 "unknowns", some of which were tentatively identified as the 3-hydroxy compounds related to the principal αβ-unsaturated 3-ketones found.

In many of the *in vivo* experiments described here this difference is explained by the fact that the samples analysed were not large enough to detect these α-ketols in the proportions described by Hechter *et al.* However, in several experiments (dog and sheep) large samples of blood containing 100–120 μg 17-hydroxycorticosterone were analysed. In the dog small quantities of a reducing substance not possessing an αβ-unsaturated 3-ketone group and different from any of those described by Hechter *et al.* (1951) were found. Otherwise no other α-ketols were found. The sensitivity of the TPTZ test in this case was checked on the same chromatogram as being < 2 μg. of α-ketol per spot. So that in terms of the content of 17-hydroxycorticosterone less than 2 per cent of each of the "unknown ketols I–V" and "VII–IX", and less than 0 5 per cent deoxycorticosterone (NaOH test limit of 0·5 μg.) were present, in contrast to a total of 32 per cent of "unknowns I–V", 1·2 per cent of "unknowns VII–IX" and 0·7 per cent deoxycorticosterone described in one protocol of Hechter *et al.* (1951). It appears possible that such ketols are only released under the conditions used by these authors. At any rate we have no evidence to prove conclusively that they are not.

The second major difference is that the isolated perfused beef adrenals of Hechter *et al.* (1951) only secreted 17-hydroxycorticosterone and corticosterone at a total rate of 1–1·5 μg./g gland/min (calculated from total α-ketol secretion rates given as 38·4–64·0 μg./min /gland of 20 g and a proportion of 54 per cent 17-hydroxycorticosterone and corticosterone after ACTH). *In vivo*, however, rates of 20–60 μg./g gland/min. were observed in cats and dogs after ACTH. Either a

large species difference exists in this respect or else these perfused glands were behaving very differently from those *in vivo*.

However, the preponderance of 17-hydroxycorticosterone and corticosterone and their approximately constant ratio of 1:1 agrees well with the results obtained in *in vivo* experiments.

The Chemical Basis of Adrenocortical Function

In Table V some of the most direct estimates of the rate of adrenal cortical secretion *in vivo* in two species under conditions equivalent to moderate or severe stress are compared. The rates of secretion are expressed in terms of the glycogenic potency of cortisone, calculated in the case of the chemical

Table V

COMPARISON OF CHEMICAL AND BIOLOGICAL EVIDENCE ON ADRENOCORTICAL SECRETION

Species	*Method of estimation*	*Rate of secretion*	*Author*
Dog	Selye-Schenker "cold-test" on adrenal venous plasma	equivalent to 5 4 mg. /kg./24 hr Compound E	Vogt (1947)
	Mouse eosinophil assay on adrenal venous plasma	equivalent to 5 mg /kg /24 hr. Compound E	Bibile (unpublished)
	Nelson's method on adrenal venous plasma (chemical)	equivalent to about 7 mg /kg /24 hr. Compound E	Nelson, Samuels and Reich (1951)
	Bush's method on adrenal venous blood (chemical)	equivalent to 4-9 mg./kg /24 hr. Compound E	Bush (1952)
Rat	Restoration of muscle work function in adrenalectomized rat	equivalent to up to 20 mg./kg /24 hr. Compound E	Ingle, Nezamis and Morley (1952)
	Bush's method on adrenal venous blood	equivalent to 4-12 mg /kg./24 hr Compound E	Bush (1951)

results on the basis of the relative potencies of adrenal steroids given by Pabst, Sheppard and Kuizenga (1947). The table thus compares the "sugar hormone" or "glucocorticoid" secretion rates determined by chemical and biological methods.

It appears from this comparison that the chemical analyses are in complete agreement with the bioassays of adrenal venous plasma for "glucocorticoids" in the dog, and are in fair agreement with the indirect estimate quoted for the rat. There is thus (1) evidence for the secretion of typical glucocorticoids by the adrenal cortex in a variety of mammalian species; (2) evidence that the substances observed chemically in adrenal venous blood are sufficient to account for all the glucocorticoid activity of adrenal venous blood.

The chemical studies so far discussed throw no light on the problem of whether the adrenal secretes a "salt-hormone". Spencer (1950) detected the equivalent of 4 μg. deoxycorticosterone per ml. of dog's adrenal venous serum, using a salt-load retention assay. But deoxycorticosterone itself is not present in dog's adrenal blood in sufficient amounts to account for this result (Bush, 1952) and since no assays of other steroids were performed it is impossible to say whether the presence of 17-hydroxycorticosterone and corticosterone would account for his results. Since cortisone acetate was found to cause sodium retention in human patients (Sprague, Power and Mason, 1950) and to have the same action on the excretion of NaCl by the dog's kidney (Roberts and Pitts, 1952) as "DOCA", it appeared to many workers unnecessary to postulate the secretion of a specific "salt hormone" by the adrenal cortex (Conn, Louis and Fajans, 1951; Conn, Fajans, Louis and Johnson, 1951). Instead it was supposed that the customary division of cortical steroids into "glucocorticoids" and "mineralocorticoids" was false (Verzár, 1952) and that the secretion of 17-hydroxycorticosterone by the adrenal cortex would satisfactorily account for the normal animal's (or human being's) capacity to regulate both mineral and carbohydrate metabolism.

However, the discovery by Grundy, Simpson and Tait

(1952*a*), using a new bioassay for "mineralocorticoid activity", that more than 80 per cent of such activity in a beef adrenal extract was contained in a substance that was confused with 11-dehydro-17-hydroxycorticosterone on most chromatograms, threw new light on the problem. This substance is probably about 20 times as active as deoxycorticosterone on a molar basis, so that physiologically significant amounts of it would have escaped detection in most, if not all, the chemical studies of adrenal venous blood that have been reported so far.

Preliminary experiments (Simpson, Tait and Bush, 1952) have indicated that a substance very similar to, if not identical with, that found in gland extracts is in fact secreted by the isolated perfused monkey adrenal, and by the dog adrenal *in vivo*. Thus two parts of the extract of a monkey's adrenal venous blood were chromatographed in parallel and chemical examination of one half of the chromatogram revealed the presence of only 1–2 μg. 11-dehydro-17-hydroxycorticosterone. After elution of the corresponding region of the untreated half of the chromatogram with ethanol the eluate was evaporated and assayed at two dilutions by the method of Simpson and Tait (1952) using the depression of the urinary $^{24}Na/^{42}K$ ratio of the adrenalectomized rat (Table VI).

The activity of this region of the chromatogram was found to be equivalent to approximately 25 μg. deoxycorticosterone acetate, whereas the amount of Compound E (estimated chemically) would only have had an activity equivalent to 0·05–0·1 μg. deoxycorticosterone acetate in this assay.

A similar analysis of dog adrenal venous blood collected *in vivo* was carried out, and in addition the "mineralocorticoid" activity of the whole chromatogram was compared with the chemical estimation of the substances detected on a parallel chromatogram. The results are shown in Table VII. Unfortunately the assay of the lower half of the chromatogram was delayed and some loss of activity was discovered by performing a second assay of the "Compound E spot". It is seen, however, that only the Compound E region of the chroma-

Table VI

BIOASSAY OF "E FRACTION" FROM AN EXTRACT OF ADRENAL VENOUS BLOOD FRACTIONATED BY PAPER CHROMATOGRAPHY (RHESUS MONKEY)

Substance injected	*Dose per rat*	*No of animals*	*$^{24}Na/^{42}K$ in urine*
10 per cent alcohol	0 1 ml	13	3 527±0 357*
"E fraction" of adrenal perfusate	1/30 total in 0 1 ml. 10 per cent alcohol	8	2 868±0 387
"E fraction" of adrenal perfusate	1/30 total in 0 1 ml 10 per cent alcohol	8	2 280±110
Adrenal extract	0 01 ml eq. in 0 1 ml 10 per cent alcohol	8	2 010±0·169
	0 02 ml eq in 0 1 ml 10 per cent alcohol	7	1 451±0·130

*S E. = $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$

Total extract equivalent to 45 min output of one adrenal from a 6 35 kg animal (♂).

Table VII

BIOASSAY OF MINERALOCORTICOID ACTIVITY IN AN EXTRACT OF DOG ADRENAL BLOOD AFTER CHROMATOGRAPHIC FRACTIONATION

Fraction number	*Region of Chromatogram*	*Chemical assay of known steroids in region*	*Expected biological activity from known steroids*	*Actual biological activity found*
1	From and including origin to edge of hydroxycorticosterone (Compound F) spot	0	0 μg DCA	0 μg. DCA
2	From and including Compound F spot to edge of cortisone spot	225 μg F	20 μg DCA	20 μg. DCA
3	Cortisone spot (Compound E)	15 μg E	1 μg DCA	56 μg. DCA
4	Corticosterone spot (Compound B)	65 μg B	10 μg DCA	<15 μg. DCA* > 0 μg DCA
5	Deoxycorticosterone	0 μg DOC	0 μg. DCA	0 μg DCA*

*Fractions 4 and 5 were tested in a different assay from 1, 2, and 3

The quantities given are equivalent to 9 0 min. output of the left adrenal (in vivo) of a 21 5 kg animal (♀)

togram contained activity that could not be explained by the compounds detected chemically. A further check was performed by acetylating part of the extract, eluting and hydrolysing the esters at the front of a chromatogram of the acetylated extract, and re-chromatographing the hydrolysed eluate. Although the recovery was low (10 per cent instead of the usual 15–40 per cent), activity was recovered from the Compound E region of the final chromatogram. This procedure demonstrated that the substance was distinct from Compound E and formed a di- or poly-acetate similar to the active substance of gland extracts.

Owing to the loss of activity in the second half of the assay it is not possible to prove the complete absence of deoxycorticosterone, but in terms of this assay (in which deoxycorticosterone is by far the most active of the chemically identified cortical steroids) it was clearly an insignificant component of the adrenal secretion, and the chemical analysis showed that less than 0·5 μg. was present per 100 μg. 17-hydroxycorticosterone.

In Table VIII an attempt is made on the basis of the actual results of this experiment to compare the physiological significance of the different components of the dog's adrenal

Table VIII

The Relative Physiological Significance of Different Components of Adrenal Secretion in the Dog in Terms of Two Known Metabolic Functions

Substance		"Sugar hormone" expressed as mg/kg/24 hr Compound E in terms of rat liver glycogen deposition assay*		"Salt hormone" expressed as mg/kg/24 hr deoxycorticosterone in terms of urinary Na/K assay†	
			percentage		percentage
Compound F		5·20	86·0	0·40	22·9
Compound E		0·23	3·8	0·02	1·1
Compound B		0·54	8·9	0·20	11·4
Compound A		0·07	1·2	0·01	—
"Salt factor"	probably	0	0	1·12	64·0
Total		6·04	99·9	1·75	99·4

*Pabst *et al* (1947)

†Simpson and Tait (1952)

cortical secretion. Since the total (chemically estimated) secretion rate of this particular adrenal was typical of the other seventeen animals examined, it is probably a reasonable appraisal, but for simplicity's sake possible synergistic effects have been neglected. It appears that few of the theories of adrenal function mentioned earlier are wholly wrong or wholly right and that, unfortunately perhaps, these results tend to support the more complicated theories rather than the simpler ones.

It appears, however, that these investigations have succeeded in limiting the field of enquiry, to some extent at any rate, for we have evidence that two important aspects of adrenal cortical function can be accounted for by chemical or partly chemical findings. There seems to be no need to postulate the secretion of any "glucocorticoids" other than those well known to chemists for some time, and similarly the "mineralocorticoid" secretion of the adrenal is probably accounted for by the secretion of the known active cortical steroids and one at present unidentified substance whose properties are well defined in some respects (Grundy, Simpson and Tait, 1952*b*).

The chemical basis of other aspects of adrenal cortical function, however, remains unknown. Gassner, Nelson, Reich, Rapala and Samuels (1951) have isolated but not identified an androgenic substance from cow adrenal blood (*in vivo*) but no evidence of progestational or œstrogenic substance in adrenal blood has been reported to date.

The most important limitation to the results and interpretations given above is that they are only strictly relevant to the secretion of the adrenal cortex at moderate to large rates and under conditions which do not pertain to the normal life of the animal. They are thus *capable* of accounting for normal adrenal physiology with regard to two sorts of function but do not exclude the possibility that the adrenal cortex in fact behaves differently in the undisturbed animal, or in animals disturbed or "stressed" in different ways. Another obvious limitation to the studies described here is that it is by no means certain that the scope of adrenal cortical function

is fully covered by the bioassays usually used for estimating or detecting "cortical activity". Thus the apparent dominance of 17-hydroxycorticosterone as a secretory product in terms of glucocorticoid activity (or of corticosterone in rats and rabbits) is only true for those aspects of adrenal cortical function in which the relative potencies of different cortical hormones are the same as in the liver glycogen deposition assay.

Again the apparent dominance of the unidentified "mineralocorticoid" in terms of Simpson and Tait's assay must not be assumed to indicate that it is the most significant secretory product with regard to *all* aspects of electrolyte metabolism. Nor is it yet known that this substance is similar in other pharmacological properties to deoxycorticosterone, or could play the rôle postulated for mineralocorticoids by Selye (1950).

It remains to discuss the large species differences in the ratio of the two major components, chemically considered, of the secretion of the mammalian adrenal cortex. The differences observed cannot be correlated with any known differences in diet, mode of life, or metabolic response to stress or to ACTH of the species examined. On the other hand, the relative constancy of this ratio in each species and the fact that it is not appreciably altered by ACTH is consistent with the theory of Hechter *et al.* (1951) that both compounds are synthesized by the adrenal from a common metabolite, probably progesterone, ACTH being supposed to act on some point of the reaction chain before the production of this common precursor. The simplest postulate available is, therefore, that these species differences in the ratio 17-hydroxycorticosterone/corticosterone are biochemically and genetically determined, and are related to different properties of one or more enzymes responsible for synthesis. An attractive possible hypothesis is that in most species the properties of the 17-oxygenating and 21-oxygenating enzymes of the adrenal cortex are such that: (*a*) their relative activity is fixed genetically and determines the ratio of the two major secretory products; (*b*) the Michaelis constants and ES dissociation velocity constants of both enzymes are considerably larger than those of *one* re-

action previous to the formation of the common precursor on which the 17-oxygenating enzyme acts.

Under these conditions, variations in the rate of synthesis of the common precursor would cause a change in total secretion rate of the two major products but no appreciable change in their ratio. The variable ratios observed in the cat could then be accounted for by a low Michaelis constant or activity of the 17-oxygenating enzyme. In this case an increase in the rate of synthesis of the common precursor above a certain limit would cause a fall in the proportion of the 17-oxygenated compound as the total output increased.

Whatever the solution of this problem the simplest hypothesis, and the only one for which there is any supporting evidence at all, is that these species differences are determined by relatively fixed biochemical properties of the adrenal cortex and not by the physiological requirements of the different species.

A very significant indication that corticosterone and 17-hydroxycorticosterone play a similar physiological rôle whether singly or in mixtures is the fact that although the rat adrenal secretes almost entirely corticosterone, 17-hydroxycorticosterone is none the less three times as potent as corticosterone in protecting the adrenalectomized rat against cold (Dorfman, 1950) and in causing liver glycogen deposition.

It is possible, however, that some functional differences will appear as a consequence of these characteristic differences in composition of the adrenal secretion.

Many observers (Dr. E. H. Kass, personal communication) have already found that cortisone is far more effective than ACTH in decreasing resistance to infections in mouse, rat, and rabbit. However, the difference is probably only a quantitative one and may be further reduced when larger and more frequent doses of ACTH are given.

Thus although these species differences are probably of more interest to the biochemist, they may have to be borne in mind by the physiologist and endocrinologist as well.

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DISCUSSION

REICHSTEIN About the possible multiple nature of the function of the adrenal gland in man—I spoke about that 2 years ago with Dr.

Thorn, who has very great experience in Addison's disease, and his opinion was that you can definitely say that there must be two functions. one which can be expressed in terms of the salt action, and the other in

improve their condition by adding small amounts of deoxycorticosterone. He estimates that the amounts necessary for maintenance of a

Then just a small point about the nomenclature. Many people think I am against using the letters of Kendall. I am not. I am against using every letter, whether it's Kendall's or mine or anybody else's. For instance, there are thousands of Compound E's in the literature, and only a very small group of people who work on the adrenal gland will know what it means. And, another point, if you use such letters, it is usual to use the name of the first one who isolated it. Cortisone would therefore be Wintersteiner's F But as soon as such a nice name as cortisone exists, one should abandon these letters They were good as long as the constitutions were not known, but once the constitutions are established and specific names are given they should disappear as quickly as possible

Pincus: In regard to the peculiar effect that Dr Bush reported in the cat, I think we should recall some data which Dr. Hechter and I published, and which are summarized in the table on p 230 This shows the mean output of the 15 α-ketols in micrograms per 20 g. of gland per hour, in a series of experiments in which ACTH was used, in which the blood was cycled through the gland only once and then

you said, probably getting a mass effect from the 17-hydroxycorticosterone during the re-cycling, whereas in the *in vivo* experiments the collection was equivalent to a single cycle.

SAMUELS: I have one question to ask Dr. Pincus about the table that he showed You had an equivalent amount of B and F in the one-cycle

Table

α-KETOLS FROM ACTH-PERFUSED BEEF ADRENAL GLANDS; OUTPUT AS MICROGRAMS PER 20 GRAMS OF GLAND PER HOUR (G. PINCUS)

Compounds	*1-cycle*	*45-cycle*	*Fresh beef gland tissue*
Unknowns I-V	294	205	70
17-OH Corticosterone	538	1155	40
Cortisone	43	205	20
Unknown VI	115		
Unknowns VII-IX	200	135	25
Corticosterone	650	408	70
Unknown X	132	33	35
11-Dehydrocorticosterone	126	105	
11-Deoxycorticosterone	65	38	?
TOTALS	2163	2284	260

stimulation, or was this simply putting it through the gland repeatedly, and not a question of continued action of ACTH?

PINCUS There was a constant content of ACTH

SAMUELS: Maintained in the blood? The ACTH would disappear from the blood rather rapidly unless it was continuously added.

HECHTER: In the one-cycle experiment we used a single dose of 25

as fast as they are formed, the possibilities of getting secondary and tertiary pathways set into action are limited.

Samuels: There is the time factor in relation to the stimulation of

Pincus No

Samuels: The single cycle is immediately after ACTH while the other involves the factor of time after initial stimulation In other words, from these experiments you can't distinguish between the question of time factor of ACTH action and the question of accumulation

Hechter: I think we can In other experiments we have passed blood containing ACTH continually through glands and repetitively removed single samples so that the time intervals involved began to approximate those described in the 45-cycle pattern The 1 1 ratio was maintained approximately throughout the entire course of the experiment

Samuels: That comes back then to the fact that as these compounds

kidney. So that I think the single cycle duplicates more closely what you get in the normal picture.

Pincus. Yes. More than the 45-cycle.

If we want to discuss the problem of whether the adrenal is producing a secretory product which has so much salt-retaining and so much sugar activity and so on, I think there are several factors which ought to be considered I would like to mention that we have conducted bio-

genetic action, in the Noble-Collip traumatic shock test, in an anti-inflammatory assay, and in the salt-retaining test.

The next situation is a little more complicated We have assayed (only for glycogenetic activity) combinations of these substances, and find that corticosterone and 17-hydroxycorticosterone and cortisone all

activity, and amongst these are Professor Reichstein's D diacetate and

also a dihydro derivative of deoxycorticosterone and a tetrahydro derivative of cortisone. These three compounds all inhibit. Adrenosterone, on the other hand, which we consider as very similar to adrenal androgen, has no effect whatsoever. So, excluding the last compound, we have some which will give you synergism, others which will give you inhibition; and how are you going to make a decision unless you know the chemical proportions of each in the secretory product?

BUSH: I think that's a very important point But you found with the principal compounds a positive synergy, and an inhibition only with D diacetate, tetrahydro-E and dihydrodeoxycorticosterone. The point I was trying to make in my paper was that the amounts of the principal steroids, estimated chemically, were sufficient to account for the glycogenic activity, as found by two different assays, even if you neglected synergy. The comparison is mainly between different species [illegible] but

[illegible]

on the dog.

PINCUS: We have found this synergism only in the glycogenetic assay.

DORFMAN: And it seems to be true both for the rat and the mouse.

REICHSTEIN: Dr. Pincus, you said that if you test the complete perfusate from a gland, you find a higher activity in every test than when you add all the known compounds. You gave the figure of two- to three-fold in the glycogen deposition test. Is this figure not much larger in the sodium retention test?

PINCUS. We ran that only once and an estimate of extent of increase can be anything you want because we don't know what the deoxycorticosterone content is. But it certainly is of a high order of activity and suggests more activity than one would expect on the basis of pure deoxycorticosterone.

IN VIVO METABOLISM OF ADRENOSTERONE

RALPH I. DORFMAN, KENNETH SAVARD and SHLOMO BURSTEIN

REICHSTEIN demonstrated the presence of 11-oxygenated C_{19} steroids in adrenal tissue (Reichstein and Shoppee, 1943). He succeeded in isolating adrenosterone and 11-hydroxy*iso*-androsterone from this source. In later studies Wolfe, Fieser and Friedgood (1941) and Dorfman, Schiller and Fish (1945) isolated an androstenolone which later was shown to be a ring C unsaturated product of 11-hydroxyandrosterone. This latter compound was shown to be present in urine as such by Mason (1945) and by Miller, Dorfman and Sevringhaus (1946).

Lieberman *et al.* (1948, 1950) at the Memorial Center, New York, have increased enormously the list of urinary 11-oxygenated C_{19} compounds in the last few years. They succeeded in isolating 11-hydroxyætiocholanolone, 11-ketoandrosterone and 11-ketoætiocholanolone. The Memorial Center group placed particular importance on the presence of 11-hydroxyætiocholanolone in human urine. They found this compound only occasionally (8 per cent of 24 subjects) in the urine of normal individuals, while this steroid was found in the urine of a high percentage (74 per cent of 35 patients) of cancer patients. It was found to be present in a relatively large number of individuals suffering from lymphatic leukæmia (100 per cent of 4 patients), hypertension (50 per cent of 6 patients), and Cushing's disease (72 per cent of 7 patients) These important findings have not been confirmed and more studies are needed to explain these striking differences in C_{19} steroid metabolism patterns

In the present study we have concerned ourselves with the relationship between C_{19} 11-oxygenated steroids from the

adrenal and those found in the urine. Adrenosterone, an adrenal androgen, was chosen for these studies. Specifically we were concerned with the possible reduction of the 11-ketone group in ring *C* to an 11-hydroxy group; the possible formation of the saturated, non-oxygenated ring *C* steroids; and finally the reduction of the Δ^4-3-ketone of adrenosterone in ring *A*. A considerable amount of work has already been done on androst-4-ene-3:17-dione and it would be of further interest to know whether the reduction in ring *A* for adrenosterone was analogous to that previously found for androst-4-ene-3:17-dione.

Subject, Materials and Methods

A 61-year-old man with rheumatoid arthritis was injected for ten days with 100 mg. of adrenosterone as a microcrystalline suspension. Urine was collected for a two-day period before treatment and during the entire period of hormone administration. The mean 17-ketosteroid excretion during the control period was 6·5 mg. per day, and during the treatment period the 17-ketosteroid excretion rose to a mean of 22·6 mg. per day, with a range of from 6·72 mg. to 44·2 mg.

Since it was expected that at least some of the urinary metabolites would be 11β-hydroxylated, and since 11β-hydroxylated steroids are easily dehydrated in the presence of mineral acids and heat, extractions were carried out under mild conditions after acid and heat. The extraction procedure is detailed in Fig 1.

Ether extracts A, B, C, and D (Fig. 1) were combined and yielded 219 mg. of 17-ketosteroid. Ether extract (E) contained 164 mg. of 17-ketosteroid. For the isolation studies, all of extracts A, B, C, D and one-half of E were available. Various aliquots were removed for additional studies so that, of the 1,000 mg. of adrenosterone administered, the urine representing 750 mg. was used for the isolation studies.

The technique used for paper chromatography is discussed elsewhere (Savard, 1952).

Experimental

The total neutral fraction was prepared in the usual way and subjected to further fractionation with Girard's reagent T. The ketonic fraction weighed 0·7 g. and the non-ketonic fraction weighed 1·6 g. The ketonic fraction was chromatographed on a 42 g. silica gel column. The residue was dissolved in 20 ml. of benzene and adsorbed on the column. The column

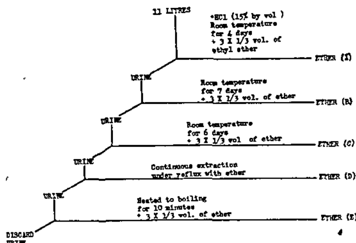


Fig. 1. Adrenosterone extraction of urine,

was eluted successively with 550 ml of benzene, 650 ml. of benzene — ethyl acetate 9.1, 100 ml. of benzene — ethyl acetate 4·1, 60 ml. of benzene — ethyl acetate 2 1, 200 ml of benzene — ethyl acetate 1:4, 50 ml. of ethyl acetate, and finally 400 ml. of ethanol Three crops of crude semicrystalline material were found in the eluates· one from the later benzene fractions; a second from the later benzene — ethyl acetate 9·1 fractions; and finally a crop from the middle benzene — ethyl acetate 2.1 fractions. These semicrystalline materials were subjected to further analysis and purification using paper chromatography.

Representative individual fractions from the silica gel column were analysed by paper strips using a ligroin — propylene glycol system. Twelve steroids were detected, eleven (I–XI) which reacted with the Zimmermann reagent on paper and substance number XII which appeared to have an α-ketol side chain. Table I gives the distribution of these steroids in

Table I

ADRENOSTERONE COMPOUNDS—PAPER ANALYSIS (ZIMMERMANN)

Silica gel col fr No	Solvent	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
4	B	+										
6, 7	B	+	+	+	+							
8	B		+	+	+							
9	B–E 9 1		+	+	+							
10–15	,,			+	+							
16	,,			+?	+?	+	+					
17	,,			+?	+?	+	+					
18	,,				+		+					
19–20	,,			+?	+	+	+					
21–22	B–E 9 1				+	+	+	+				
23–24	B–E 4 1					+		+				
26–29	B–E 2·1					+	+	+	+	+	+?	
30	,,					+?		+	+	+	+	
31	,,							+	+	+	+	
32	,,							+	+	+	+	
33–35	,,							+	+	+	+	
36–40	,,							+	+	+	+	+
41–46	B–E 1 4							+	+	+	+	+

B = Benzene E = Ethyl Acetate

the various fractions of the silica gel column. Since many fractions from the silica gel column contained more than one component, the material was run again on preparative papers so that approximately 20–35 mg. of material was placed on paper sheets 15 cm. wide. The single steroid zones were cut out, eluted with methanol and the various compounds crystallized. Identifications were made on the basis of paper chroma-

tography, melting points, melting points of mixtures with authentic samples, and finally infra-red analysis.

Table II lists the compounds that were isolated. Eight compounds were identified.

Table II
METABOLITES OF ADRENOSTERONE
(750 mg)

Steroid	*mg Isolated*	*Per cent Conversion*
11-Hydroxyandrosterone Androst-9(11)-en-3α-ol-17-one	42 7	5 7
11-Hydroxyetiocholan-3α-ol-17-one Ætiochol-9(11)-en-3α-ol-17-one	5 7	0 8
11-Ketoandrosterone	4 9 (crude)	0 7
11-Ketoætiocholan-3α-ol-17-one	3 0	0 4
Androsterone	Trace	
Epiandrosterone	Trace	

Discussion

The principal metabolite of adrenosterone was 11-hydroxyandrosterone, which was isolated in a yield of 5·7 per cent. 11-Hydroxyætiocholan-3α-ol-17-one was also found, but only to the extent of 0·8 per cent From these findings and previous findings with androst-4-ene-3.17-dione there is the indication that the presence of the 11-keto group tends to direct the reduction in ring *A* more toward the androstane than the etiocholane configuration. In an *in vivo* study (Dorfman, Wise and Shipley, 1950) of androst-4-ene-3 17-dione it was found that essentially equal amounts of androsterone and ætiocholan-3α-17-one were isolated This finding has been confirmed using deuterium-containing steroid (Gallagher *et al.*, 1951).

The finding of 11-hydroxyandrosterone and 11-hydroxyætiocholan-3α-ol-17-one clearly inidcate the reduction of the 11-keto group to the 11β-hydroxy group. Similarly Mason

(1950) isolated 17-hydroxycorticosterone after cortisone treatment and Dobriner (1951) isolated 11-hydroxyætiocholan-3α-ol-17-one after cortisone administration to a normal man.

We have no indications that adrenosterone gives rise to non-oxygenated saturated ring *C* steroids such as androsterone or ætiocholan-3α-ol-17-one. Only a trace of androsterone was detected by infra-red spectroscopy. Ætiocholan-3α-ol-17-one has thus far not been detected. Actually it appears that the endogenous production of these steroids has been suppressed.

Part of the 11-hydroxy steroids were isolated as their corresponding Δ^{9}-unsaturated derivatives (Table II). This was expected since the compounds were subjected to hydrochloric acid and heat. It is unlikely that these unsaturated steroids represent true metabolites.

Summary

11-Hydroxyandrosterone, 11-hydroxyætiocholan-3α-ol-17-one, 11-ketoandrosterone, and 11-ketoætiocholan-3α-ol-17-one have been shown to be metabolites of adrenosterone. The presence of the 11-oxygen function appears to direct the reduction of ring *A* to the androstane configuration. The reduction of the 11-keto group to the 11β-hydroxy group is clearly demonstrated.

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CURRENT STATUS OF CORTICOSTEROID METABOLISM IN MAN*

G. PINCUS, E. B. ROMANOFF and LOUISE P. ROMANOFF

Introduction

THE objective of this paper is to present recent analytical data on the corticosteroids present in certain human tissues and fluids and to attempt to assess the significance of the similarities and differences observed. It should be stated at the outset that the nature of some of our findings necessitates caution in drawing any conclusions; the complexity of the corticosteroid patterns observed and the limited sources of material drawn on for the analytical work give a very preliminary flavour indeed to the nonetheless laboriously assembled data.

Our original intentions were comparatively simple. The fortunate availability of human adrenal glands, taken at bilateral adrenalectomy of patients with malignant hypertension and of other patients with inoperable cancer of the breast and of the prostate, led us to undertake the perfusion *in vitro* of these glands following the techniques which have been employed so successfully in our laboratories with the isolated adrenal glands of other species (cf. Hechter *et al.*, 1951). Since we had already shown that methods of paper chromatography permitted the assessment of a rather regular pattern of corticosteroid secretory product from bovine glands following their perfusion with ACTH (Pincus, Hechter and Zaffaroni, 1951) we proposed applying similar analytical methods to perfusates from human adrenal glands. The secretory products so

*Investigations described in this paper have been aided by grants from the U.S. Public Health Service, the U.S. Atomic Energy Commission and the Williams-Waterman Fund of the Research Corporation.

obtained were to be compared with the corticosteroids of human urine analysed by similar methods. As a site for the intermediate metabolism of corticosteroids in man we selected the knee joint, from which it is not too difficult to withdraw samples of synovial fluid following corticosteroid injection.

The results of our experiments resolved themselves into a much less simple picture than we had expected. The presence of a large variety of steroids was indicated in each source examined.

Subjects and Methods

Urinary steroids were obtained from twenty-four-hour specimens collected from schizophrenic, arthritic and normal healthy subjects of both sexes. The analyses were conducted usually with the pooled extracts of two such twenty-four-hour specimens. The methods for extraction have been recently described (Romanoff, Wolf and Pincus, 1952), and involved incubation of the fresh urine with spleen glucuronidase, extraction of the lipids with methylene chloride, separation of the neutral lipids, their fractionation by elution from silica gel into three principal fractions—(*a*) the less polar, (*b*) the more polar, and (*c*) the most polar—and the resolution by paper chromatography of the steroids contained in fractions (*a*) and (*b*).

The adrenal glands perfused came from patients with malignant hypertension (supplied by Dr. George Thorn of Harvard University) and with prostatic or breast carcinoma (supplied by Dr Charles Huggins of the University of Chicago). The method of perfusion employed was that originally described for beef adrenal glands (Hechter *et al.*, 1951). Following the perfusion of a control sample of blood, ACTH was added to the medium (in amounts indicated in the text) and the ACTH-stimulated gland perfusate was then collected over periods ranging from one to four hours. The steroids were extracted as previously described (Hechter *et al.*, 1951), and the neutral lipid fractions eluted from silica gel which contain the hormonal steroids and related compounds, were chromatographed

on paper using the propylene glycol-toluol system of Zaffaroni *et al.* (1950).

The synovial fluids analysed were collected at twenty-four hours following a single injection of 1 ml. of a 25 mg. per ml. microcrystalline suspension of 17-hydroxycorticosterone. The neutral lipids were extracted directly with methylene chloride and were not fractionated on silica gel, but analysed directly on paper, since various pigments and non-specific substances ordinarily removed by silical gel chromatography apparently were absent from these extracts.

Results

(a) Urine Extracts

Urine specimens from 28 subjects were analysed to a greater or lesser extent by the foregoing methods. They were obtained from four groups, as indicated in Table I, which also lists the mean concentration of formaldehydogenic substance (FS) and

Table I

MEAN FORMALDEHYDOGENIC SUBSTANCE (FS) CONCENTRATION FOLLOWING GLUCURONIDASE HYDROLYSIS OF URINE SAMPLES FROM VARIOUS SUBJECTS

Type of subject	*No*	*Mean age years*	*Mean FS mg /24 hr*	*FS range mg /24 hr*
Young normal	6	27·5	8·65	5·5–14·5
Older normal	11	74·2	3·19	2·0– 6·1
Arthritic	5	55·2	4·38	2·5– 7·7
Schizophrenic	5	30·6	4·52	2·9– 9·1

the range of values obtained. There appears to be a lower output in the aged normal subjects and, as we have previously noted using other methods of determination (Pincus, 1952*a*), a lower output in schizophrenic subjects of the same age as normal, healthy subjects.

Assuming that the FS obtained represents chiefly steroid ketol (and perhaps glycol) we have attempted to resolve the obvious mixtures. Working initially with pooled specimens from normal subjects we have found that silica gel eluate

containing the less polar compounds contains 16 substances, none of which have we been able to identify with certainty. These include 10 compounds having α-ketol side chains (Nos. 5A, 5B, 6, 7, 8, 9, 11, 12, 12A, and 14) of which two (Nos. 5A and 5B) give reactions characteristic of αβ-unsaturated ketones. Of the remaining 6 compounds, three react like 21-carbon steroids lacking the ketol side chain (Nos. 3, 4 and 13) and three react like 19-carbon 17-ketosteroids (Nos. 1, 2 and 10).*

We have examined these less polar silica gel eluates of the urinary extracts from four normal and six schizophrenic subjects for the presence or absence of these 16 compounds. The data are presented in Table II. Ten of these compounds (Nos.

Table II

"CORTICOIDS" OF HUMAN URINE (EARLY SILICA GEL ELUATES)

Compound number	*Present (+) in*	
	Normals (4)	*Schizophrenics (6)*
14	+	+ + +
13	+ + + +	+ + + + + +
12A	— — — —	+ + +
12	+ +	+ + +
11	+ + + +	+ + + + + +
10	+ + + +	+ + + + + +
9	+ + + +	+ + + + + +
8	+ +	+ + + + +
7	+ + + +	+ + + + + +
6	+ + + +	+ + + + + +
5B	+ + +	+ + + + + +
5A	+ + + +	+ + + + + +
4	+ + +	+ + + + + +
3	+ + + +	+ + + + + +
2	+ + + +	+ + + + + +
1	+ + + +	+ + + + + +

1, 2, 3, 5A, 6, 7, 9, 10, 11 and 13) have appeared invariably in all extracts analysed, one (No. 12A) has not been seen in any normal subjects' extracts, but has appeared in half of the

*It should be noted that these compounds are numbered in the order of their running rates on paper, No. 1 being the fastest running and No. 14 the slowest.

schizophrenic subjects' samples; two (Nos. 4 and 5B) have been observed in every one of the schizophrenics' samples and in three of the four normals; the remaining three (Nos. 8, 12 and 14) have been observed in a greater or lesser number of instances but not all specimens from either type of subject. It should be pointed out that, on a qualitative basis, the presence of a given compound is more significant than its absence, since an absence may merely indicate a quantity too small to identify by the methods we have used. The spectrographic methods employed are, on the other hand, sensitive to 10 to 20 μg. amounts of steroid.

In the more polar silica gel eluates we have detected the presence of 17 compounds, all α-ketols, of which five have been identified with certainty, namely (in decreasing order of running rates): corticosterone, 17-hydroxy-11-dehydrocorticosterone, 17-hydroxycorticosterone, pregnane-3α:17α:21-triol-11:20-dione, and pregnane-3α:11β:17α:21-tetrol-20-one. Nine of the compounds observed have running rates on paper faster than cortisone, and while within any single chromatogram they are distinguishable one from the other, it is a little difficult to compare with certainty any single substance from chromatogram to chromatogram. Accordingly we have listed in Table III the mean frequency of appearance of identifiable substances in three groups of subjects. The two most rapidly running compounds have not been observed in the extracts from arthritics' urines but they appear in about half of the

Table III

"CORTICOIDS" OF HUMAN URINE (SUBSTANCES IN LATE SILICA GEL ELUATES WITH RUNNING RATES FASTER THAN CORTISONE). TOLUENE . PROPYLENE GLYCOL SYSTEM ON PAPER

Running time on paper	*Total No. Cpds*	*Average No of Compounds in*		
		Normals (11)	*Arthritics (4)*	*Schizophrenics (6)*
24 hr.	4	2 0	2·0	3·3
8 hr.	3	2·0	2·3	2·2
4 hr	2	0·8	0·0	1 0

extracts from the other subjects. The three less rapidly running compounds appeared in practically all of the arthritic subjects' extracts, but somewhat less frequently in the others analysed. The four slowest running compounds appeared frequently in schizophrenic subjects' extracts, least frequently in the arthritics'.

In Table IV are listed the eight compounds having running rates of cortisone or slower and their relative frequency of occurrence in the three types of subjects. Three of these

Table IV

"CORTICOIDS" OF HUMAN URINE (SUBSTANCES IN LATE SILICA GEL ELUATES WITH RUNNING RATES OF CORTISONE OR SLOWER)

Compound	*Solvent System on Paper*	*Percentage of Subjects Excreting Compound*		
		Normal (11)*	*Arthritic* (4)*	*Schizophrenic* (6)*
No. 29	Benzene Formamide	30 (10)*	0	100
No. 28	,,	80 (10)*	100	100
Pregnane-3 11:17,21-tetrol-20-one	,,	100	100	100
Pregnane-3 17 21-triol-11 20-dione	,,	100	100	100
No. 27	,,	64	75	0
17-Hydroxy corticosterone	Toluene Propylene Glycol	100	100	17
No 26	,,	73	100	100
Cortisone	,,	100	100	100

*( )—No of subjects' urines analysed

compounds, cortisone, its tetrahydro derivative (pregnane-3. 17:21-triol-11:20-dione) and the corresponding derivative of 17-hydroxycorticosterone (pregnane-3:11:17:21-tetrol-20-one) have been observed in all specimens analysed. Compounds 26 and 28 have been observed in all specimens from arthritic and schizophrenic subjects and in most of the normal

subjects' specimens. 17-Hydroxycorticosterone has been observed in all specimens from normal and arthritic subjects, but appeared in only one of the six schizophrenics' urine extracts. Of the remaining two compounds, No. 27 has not been observed in any schizophrenics' urine extract, but it has been seen in 2/3 to 3/4 of the other specimens, and No. 29 has occurred in all schizophrenic subjects' urines, but has been seen in only three of the fourteen other extracts analysed.

In summary, of 33 substances identifiable by our analytical methods 17 have appeared consistently in all types of urine specimens analysed, whereas 16 are absent to a greater or lesser degree depending upon the type of subject examined.

(b) Human Adrenal Perfusates

In a previous publication (Pincus, 1952*b*) I have presented data indicating that ACTH will, under certain conditions, stimulate the isolated perfused human adrenal gland to an increased output of FS. Employing these conditions, we have perfused six glands taken freshly at operation and analysed the neutral lipid silica gel eluates of three control perfusates (without ACTH) and six perfusates following ACTH administration. The results of our analyses are shown in Table V, in which the substances observed are classified on the basis of their relative running rates and their specific reactions. The relative running rates are calculated in terms of the rate of 17-hydroxycorticosterone, the running rate of which is taken as 1·0. The compounds observed are described according to the "type" reactor: thus, "Tetrahydro"-type compounds are substances reacting as α-ketols, but giving no indication of the presence of a ketone in ring *A*; "Cortisone"-type are α-ketols with αβ-unsaturated ketones; "Dihydro"-type are α-ketols with ring *A* saturated and reacting as 3-ketones; "Progesterone"-type are C_{21} compounds lacking the α-ketol reaction but giving reactions as αβ-unsaturated ketones; "Pregnanedione"-type are non-ketols with a 3-ketone but not αβ-unsaturated; "Glycol"-type are compounds which are non-ketols, but reacting like 20.21-diols.

Table V

"CORTICOIDS" IN PERFUSATES FROM HUMAN ADRENAL GLANDS TOLUENE PROPYLENE GLYCOL SYSTEM ON PAPER

Running Rates	*"Tetrahydro" type*		*"Cortisone" type*		*"Dihydro" type*		*"Progesterone" type*		*"Pregnanedione" type*		*"Glycol" type*		*All types*	
	*Cont**	*ACTH*	*Cont*	*ACTH*	*Cont*	*ACTH*	*Cont*	*ACTH*	*Cont*	*ACTH*	*Cont*	*ACTH*	*Cont*	*ACTH*
0 05–0 8	1	2	2	3	2	2	1	2	2	2	1	1	9	12
0 9–2 5	2	3	1	1	1	1	1	1	1	1	1	1	7	8
2 6–10	2	2	1	2	1	1	0	1	1	2	0	0	5	8
11–30	2	2	0	1	0	0	0	1	0	0	0	0	2	4
31–100	1	2	2	2	0	1	2	2	1	2	0	0	6	9
Totals	8	11	6	9	4	5	4	7	5	7	2	2	29	41
α-Ketols	18	25												
Non-ketols	11	16												

*Controls.

The data of Table V inform us of the types of compounds with these running rates observed in perfusates from control and ACTH-stimulated glands. Thus eight α-ketols of the tetrahydro type were observed in control perfusates, whereas 11 such compounds were seen in the ACTH perfusates. Similarly, the controls' output of "cortisone"-type compounds was six, and the ACTH perfusates contained nine. Only among the glycols was there no increase of total numbers of compounds in the ACTH perfusates. The indications of Table V are that 29 different compounds were seen in control perfusates and 41 following ACTH; of these, 18 in the control perfusates were α-ketols and 11 non-ketols, whereas following ACTH there were 25 and 16 respectively.

Actually the frequency of occurrence of the compounds listed was not the same from perfusate to perfusate. In Table VI we list the compounds by number and the frequency of occurrence of each. Only four compounds (Nos. 7, 20, 21 and 40) were observed in every control and every ACTH perfusate examined Compounds 17, 31 and 33 were observed in each of the three control perfusates but in only 1/2 to 2/3 of the ACTH perfusates. Compounds 6, 24, 28 and 37 were seen in all ACTH perfusates but not in all the controls analysed, and compounds 29 and 41, seen also in all ACTH perfusates, were observed in none of the control samples. All of the 28 remaining compounds were observed in 1 to 5 of the ACTH perfusates, but 10 of them were not seen in control perfusates.

For the most part we have been unable to identify the compounds listed in Table VI. By all criteria, compound No. 7 appears to be 11-deoxycorticosterone; compound No. 11 is probably corticosterone; compound No. 21 is almost undoubtedly Reichstein's Compound P (*allo*pregnane-3:17:21-triol-20-one); No. 26 is 17-hydroxycorticosterone and No. 40 is probably a tetrahydro derivative of No. 26. On the basis of Zaffaroni's classification, the probable numbers of oxygen atoms are suggested in Table VI, but these are at best approximate.

Table VI

RELATIVE FREQUENCY OF OCCURRENCE OF "CORTICOIDS" IN CONTROL AND ACTH-PERFUSED HUMAN ADRENAL GLANDS

Running rate	*Colour No*	*Reaction type*	*Control (3)* Frequency of occurrence*	*ACTH (6)* Frequency of occurrence*	*Standard compounds*
81-100	1	"Pregnanedione"	1	2	
	2	"	0	1	
	3	"Progesterone"	1	2	
	4	"	2	1	11 Deoxy-corticosterone
$(0_2–0_3)$†	5	"Dihydro"	0	2	
	6	"Cortisone"	2	6	
	7	"	3	6	
	8	"Tetrahydro"	1	3	
	9	"	0	4	
11-30	10	"Progesterone"	0	1	Corticosterone
$(0_3–0_4)$	11	"Cortisone"	0	3	Δ^4-Pregnene-17 21-diol-3 20-dione
	12	"Tetrahydro"	2	3	
	13	"	1	1	
2 6–10	14	"Pregnanedione"	1	2	
	15	"	0	1	Pregnane-17 21-diol-3 11 20-trione
	16	"Progesterone"	0	4	
$(0_4–0_5)$	17	"Dihydro"	3	3	
	18	"Cortisone"	2	5	*allo*Pregnane-3 17 21 triol-20-one
	19	"	0	1	
	20	"Tetrahydro"	3	6	
	21	"	3	6	
0 8–2 5	22	"Glycol"	1	3	
	23	"Pregnanedione"	1	3	Pregnane-3 17 21-triol 11 20-dione
$(0_4–0_6)$	24	"Progesterone"	1	6	
	25	"Dihydro"	2	1	
	26	"Cortisone"	2	1	17-Hydroxycorticosterone
	27	"Tetrahydro"	1	2	
	28	"	2	6	Cortisone
	29	"	0	6	
0 05–0 8	30	"Glycol"	1	3	
$(0_6–0_7)$	31	"Pregnanedione"	3	3	
	32	"	2	1	
	33	"Progesterone"	3	4	
	34	"	0	2	
	35	"Dihydro"	1	2	Tetrahydro-cortisone
	36	"	1	1	
	37	"Cortisone"	2	6	
	38	"	2	4	
	39	"	0	4	
	40	"Tetrahydro"	3	6	
	41	"	0	6	

*( ) = No of subjects analysed
†Probable number of oxygen atoms

In summary, in the control perfusions seven compounds were seen consistently in all samples, five of them α-ketols, one an αβ-unsaturated ketone of the progesterone type and one a non-ketolic saturated 3-ketone. In the ACTH perfusates ten compounds were seen consistently in all samples, nine of

which are α-ketols and the tenth an αβ-unsaturated ketone of the progesterone type.

(c) The Intra-Articular Injection of 17-Hydroxycorticosterone

From the knee joints of five arthritic patients receiving 17-hydroxycorticosterone twenty-four hours earlier by intra-articular injection, synovial fluid was withdrawn and analysed by paper chromatography. The findings are presented in Table VII, which lists the substances seen in order of their relative running rates and as compound types. A total of 25 different substances were observed one or more times. Among them only three substances, and all of them α-ketols of the "dihydro"-type, were seen consistently in all samples; five compounds were seen in four of the five samples; the remaining 16 compounds were observed in one to three samples. Of the 25 substances 18 are α-ketols and seven are non-ketols, but no glycols were observed. 17-Hydroxycorticosterone was definitely identified in three of the five specimens. No other compounds have been with certainty identified, but reduced ring *A* transformation products are strongly suggested.

Discussion

The following is a summary of the total "corticoids" found in urine extracts, in perfusates from isolated ACTH-stimulated human adrenal glands and in synovial fluid following 17-hydroxycorticosterone administration:—

Urine: 29 α-ketols, of which 11 are αβ-unsaturated ketones; 4 non-ketols, of which 2 are αβ-unsaturated, one a glycol.

Perfusates: 25 α-ketols, of which 9 are αβ-unsaturated ketones; 16 non-ketols, of which 7 are αβ-unsaturated ketones, 2 glycols.

Synovial Fluid: 18 α-ketols, of which 5 are αβ-unsaturated ketones; 7 non-ketols, of which 2 are αβ-unsaturated, no glycols.

There are a number of puzzling discrepancies, particularly between the urinary and perfusate findings. Thus, there are two more αβ-unsaturated ketolic ketones in urine than in the

Table VII

"CORTICOIDS" OBTAINED FROM HUMAN SYNOVIAL FLUIDS 24-HOURS AFTER THE INTRA-ARTICULAR INJECTION OF 17-HYDROXYCORTICOSTERONE. ANALYSES OF FIVE SAMPLES

Relative running rates	*Types of compound*									
	"Tetrahydro"		*"Cortisone"*		*"Dehydro"*		*"Progesterone"*		*"Pregnanedione"*	
	No	*No times seen*	*No*	*No times seen*	*No*	*No times seen*	*No*	*No times seen*	*No*	*No times seen*
0 05–0 8	1	3	2	2 4	2	5 5	2	1 1	2	1 1
0 9–2 5	1	2	1*	3	1	4	0	0	1	2
2 6–10	1	4	1	1	2	3 5	0	0	1	4
11–30	1	1	0	0	1	3	0	0	0	0
31–100	1	3	1	2	2	4 1	0	0	1	1
Totals	5	—	5	—	8	—	2	—	5	—

*17 Hydroxycorticosterone

adrenal perfusates. And in the perfusates we have observed 16 non-ketols whereas in the urine extracts only four have been seen. Judging by the effects of synovial tissue upon 17-hydroxycorticosterone we would expect reduction of ring *A* and of the C-21 alcohol to be clear metabolic events and the resultant compounds should appear in the urine. That some do is obvious, but it is clear that four non-ketolic synovial fluid metabolites and 13 non-ketols of the adrenal perfusates are not present in the urine extracts. Eighteen ring *A* reduced ketols appear in urine and 16 have been observed in perfusates, 13 in synovial fluid metabolites, and here the suggestion of the appearance of metabolites of presumed adrenal products is slight indeed.

The central problem of our data involves the steroids from the perfusates. Are they true secretory products? The consistent appearance of 15 α-ketols in the perfusates of ACTH-stimulated bovine adrenals is not replicated here. Only nine α-ketols were regularly seen in all similar human adrenal perfusates; if we include those ketols which occurred in four or five of the samples analysed, the total rises to thirteen. Do the additional twelve seen with less frequency represent chance metabolites formed under the influence of catabolic systems in the adrenal tissue or the blood itself? Since these glands were taken from diseased persons, is the peculiar array in fact due to the diseases themselves? Moreover, the predominance of corticosterone and 17-hydroxycorticosterone seen in bovine adrenal perfusates is not seen here. In fact, in a number of perfusates both of these compounds are absent.

We have repeatedly stated that the perfused, isolated adrenal cannot be claimed as the secretory organ at work *in vivo*. It may be that the abnormal perfusion conditions lead to the production of substances not ordinarily produced *in vivo*. It is interesting, however, that the array of urinary corticosteroids is, if anything, greater than that seen in the perfusates and that the number of metabolites of a single corticosteroid (17-hydroxycorticosterone) from a single tissue

(synovium) approaches the number of substances seen in urine.

Our data clearly present us with a number of unsolved problems. Most essential is the positive identification of all of the substances observed in urine, in perfusates, in synovial fluid. Until we know what these substances are in fact we have no firm basis for anything but the most tentative speculation. Secondly, an examination of the perfusates of "normal" human adrenal glands seems most desirable. This we hope to obtain from glands taken at autopsy from accident cases. Thirdly a comparison should be made of the adrenal venous steroid content *in vivo* with the perfusion products; this we hope to do by means of cannulation of the renal vein in selected subjects. Fourthly, the synovial fluid data which we have presented require amplification in a number of directions; we need to work with synovial fluid *in vitro* as well as *in vivo*, and the effects upon a variety of corticosteroids should be studied. Fifthly, the urinary corticosteroid variations following the administration of active hormone should give us some indication of the probable origins of some of the compounds seen, and indications also as to the appearance and non-appearance of certain compounds in certain disease states. Finally, the data here presented have been merely qualitative. We are in the process of establishing methods for the accurate quantitative analysis of the various chromatogram constituents. With the proper application of such methods the significance of numerous variations should be made clear.

I have had the privilege of presenting to you a complex of analytical data which is at best suggestive. It represents the defeat of some fairly simple expectations and the beginning of what must be a very laborious effort indeed. Well, all inquiries of this sort must have a beginning From such a beginning, the application of careful techniques and patient analysis is bound to lead to enlightenment. Perhaps when we meet again the mysteries presented will be solved, the errors uncovered and the extraordinary steroid ubiquity explained.

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DISCUSSION

GRAY: We have been doing work rather similar to that of Dr. Pincus, largely on the urine of patients with diabetic pregnancy. We divide our material into four fractions. one containing free steroids extractable with chloroform at pH 7, together with steroids that are easily hydrolysable, and extractable with chloroform after acidification at pH 1; an-

found only 15 compounds, but I think that agrees fairly well with Dr. Pincus, because we've been concerned essentially with the more polar compounds. Nine of them have αβ-unsaturated ketone groups, and 5 have reducing

group. We fee

tetrahydro-E.

a re-

onidase hydrolysed urine. There's a lot of tetrahydro-compounds

and usually some material which may be dihydro-Compound E. There is no X_4 in this fraction, but there is another $\alpha\beta$-unsaturated ketone, X_4, which again appears to be present in the last half of the menstrual cycle and appears to crop up in pregnancy.

I think it's interesting that although we have used a very different system for the analysis from that used by Dr Pincus—we have used Bush's system—we get much the same sort of pattern.

FINKELSTEIN We have observed by means of fluorimetry, by a reaction using phosphoric acid, that the neutral water-soluble fraction

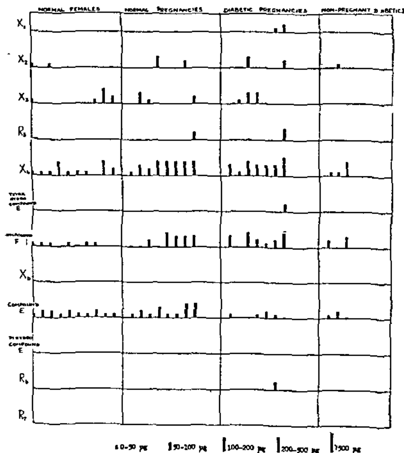


FIG. 1. Pattern of excretion of free and readily hydrolysed *steroids (C H. Gray).*

of every male urine contains a substance which is excreted in amounts of about 1 mg./l. urine, and which seems to be different from any other substance detectable until now. We have found the same substance in female urines. It gives a marked peak at about the 10th day of the menstrual cycle, and sometimes also at about the 20th day of the cycle, and is usually present during the whole cycle. By means of fluorimetry we also found that this substance was excreted by two female pseudohermaphrodites, at about the rate of 4–5 mg./l. urine. In late pregnancy about the same amount was obtained. We have also seen that after

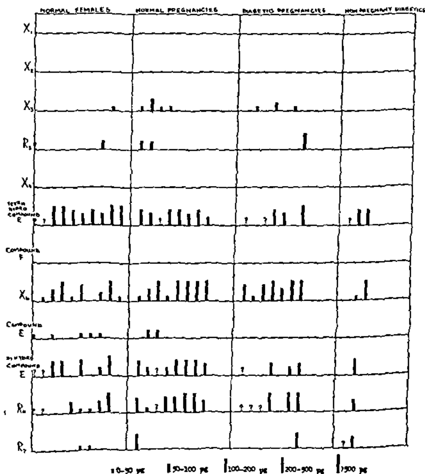


FIG. 2. Pattern of excretion of steroids conjugated with glucuronic acid (C. H. Gray)

injection of ACTH the excretion was elevated for two or three days. We collected and fractionated the urine of the pseudohermaphrodites and have asked Prof. Reichstein whether he can tell us what it is Prof Reichstein said he believes it is a new substance. Perhaps he would say something about it now.

REICHSTEIN: I received an extract from Dr Finkelstein and Prof.

urinary steroid of their collection. We have done only preliminary

glucuronidase hydrolysis of urine gives reproducible results? The

have analysed, particularly for the most polar compounds (that is, the group which includes cortisone and 17-hydroxycorticosterone), the patterns are remarkably similar from urine to urine, for example from

of a single individual a number of times—that variability is less.

MARRIAN I suspect there may be glucuronidase inhibitors in urine in variable amounts The other question I wanted to ask was, when you extracted with methylene chloride after glucuronidase hydrolysis, did you wash the extracts with alkali and water?

PINCUS Yes, very rapidly.

MARRIAN And the third point, about these unknown compounds, I wonder whether you aren't perhaps putting too much faith in the Zaffaroni spot tests Do you really feel convinced that all these 41 compounds are steroids?

PINCUS I regard them as spots on paper that give certain colour reactions Until the compounds are identified by classical methods, I will continue to say that.

SAMUELS· I wanted to ask Dr Pincus if he had run the urines of

untreated Addisonians or the adrenalectomized individuals. It wo be interesting to see how many of these compounds are missing in th type of case.

PINCUS: We have pleaded with both Dr. Thorn and Dr. Huggi but it's almost impossible. One patient was taken off all medicati for two days and we have the urines, but that was a fairly recent ca and I can't tell you anything about it.

SAMUELS: One of the men in our laboratory, applying Dr. Nelso technique to urine samples, has found that Addisonians give ze ... day ... re is ... low ... e th

with chloroform is adjusted to pH 4 8 and placed in the incubat ... fo

PINCUS. We have applied exactly the same analysis to urines extra ... as we can estimate ... lerivatives increase ... reased only slightly but we think they may increase somewhat. Again, the quantitativ ...

instead of the equivalent of 48 hours urine we take the equivalent o more than 100 hours, then we can see a fair replica of the chromato ...

GRAY: We could not detect Compound F in the glucuronidase ...

instance, might be because they are kept inside instead of walking around?

PINCUS: No. These are working schizophrenics.

VENNING: I would like to make another comment in connection with the metabolism of adrenocortical steroids. We have had the opportunity of examining the corticoid content of the urine of a patient with pemphigus who has been receiving 1,000 mg of cortisone a day. We could only account for 0·3 per cent of the active substance in the urine;

that we've actually been able to isolate and identify was 2·5 per cent: 1·5 per cent as 11-hydroxyætiocholanolone, and 1 per cent as 11-keto-

DJERASSI: One thing that impresses me is that all the saturated adrenal steroids which were isolated by Reichstein, Kendall and Wintersteiner were *allo*pregnanes, while the few that have been identified so far from urines are all pregnanes. Furthermore, I assume that your tetrahydro E and F are pregnane derivatives rather than *allo*-pregnane derivatives—they are not Reichstein's Compounds V and D? Which configuration do they have at 3 and at 11?

PINCUS: 3α 11β.

DJERASSI: They were identified with the known compounds?

REICHSTEIN: I am just trying to get a little more of this. The simplest way would be to synthesize it. Perhaps Djerassi has done that already.

DJERASSI: Few people realize that your Compound C is the only remaining known adrenal steroid which has not been synthesized partially or totally. The question is whether it is worth the effort, because it is not too simple a task.

REICHSTEIN: About a week before I left Basel we obtained some of this compound, we wanted to separate a new batch of adrenal

l out, but puri-

ation which was

as tested in the

be best to synthesize the compound

FIESER: I was just wondering if some of the studies of steroid metabolites may be confused by the formation of molecular complexes, similar to the type that I mentioned the other day in my talk on the oxidation of cholesterol. I found that 3α-hydroxy steroids, specifically the type of epicholesterol, form complexes with 3-ketones, 1:1 complexes that crystallize very nicely, and sometimes are not resolvable by chromatography. The complex between 6β-hydroxycholestenone and epicholesterol, which I mentioned, is separable on a chromatograph column. But I also ran into a complex, as a result of an oxidation, between epicholesterol and cholestenone, and this was apparently homogeneous on an alumina column, the two components coming off together. And when one is dealing with mixtures that contain 3α-hydroxy steroids and various ketones, this sort of complex formation might obscure the issue.

ESTIMATION OF INDIVIDUAL ADRENOCORTICAL HORMONES IN HUMAN PERIPHERAL BLOOD

C. J. O R. MORRIS and D. C. WILLIAMS

A METHOD for the estimation of the adrenocortical hormones in blood would obviously be of great value both for physiological and clinical studies. The estimation of these substances and their metabolites in urine is complicated by difficulties in the hydrolysis of their conjugates, a problem which has not yet been satisfactorily solved. The estimation in blood is more direct and the work of Nelson and Samuels (1952) appears to indicate that at least the greater part of these hormones occurs in blood in the free state.

The first attempt at a method for the estimation of the adrenocortical hormones in human peripheral blood appears to be that of Corcoran and Page (1948) who adapted their method for urinary formaldehydogenic steroids to blood. More recent studies have shown that their values were very much too high, presumably due to non-specific interference. Several workers have investigated the adrenal venous effluent of experimental animals, particularly after stimulation with adrenocorticotrophic hormone. Thus Reich, Nelson and Zaffaroni (1950) were able to isolate 17-hydroxycorticosterone from the adrenal venous blood of dogs stimulated with ACTH. The problem of the determination in peripheral blood, which is essential for studies in human beings, is rather more difficult owing to the very low levels. Bush (1952) has fractionated blood extracts by paper chromatography and his methods seem very well suited to qualitative investigations, although quantitative estimation by such methods is rather more difficult. Nelson and Samuels (Nelson *et al.*, 1951, Nelson and Samuels, 1952) have developed an excellent method for blood

17-hydroxysteroids based on chromatography on a magnesium silicate-Celite column followed by estimation by a micro Porter-Silber reaction. This reaction is believed to be specific for substances with a 17-hydroxyketol side chain and would therefore estimate only Compounds E and F. Nelson and Samuels (1952) examined their 17-hydroxysteroid fraction by paper chromatography using the method of Zaffaroni, Burton and Keutmann (1950) and concluded that it contained only Compound F. They found an average normal range of 4–10 μg. of 17-hydroxysteroids/100 ml. of blood.

The purpose of the present work was to devise a method capable of separating and estimating Kendall's Compounds A, B, E and F (11-dehydrocorticosterone, corticosterone, 11-dehydro-17-hydroxycorticosterone, and 17-hydroxycorticosterone) and Reichstein's Substance S (17-hydroxy-11-deoxycorticosterone) in reasonable quantities of human peripheral blood. This has been achieved by a combination of partition chromatography and polarographic estimation as the Girard hydrazones of the Δ^4-3-ketosteroid group common to all these substances.

Heparinized blood was used and the plasma separated as soon as possible. Twenty ml. of plasma were usually used. The course of extraction and preliminary fractionation are shown in Fig. 1.

The plasma proteins are precipitated by the addition of 3 volumes of ethanol. The precipitate is separated by centrifugation, washed with ethanol, and the combined extracts concentrated *in vacuo* and made up to 20 per cent ethanol This is extracted three times with ethyl acetate and the combined extracts evaporated to dryness *in vacuo*. The residue is dissolved in 20 per cent ethanol and stored at −10°C. overnight. Next day the mixture is centrifuged to remove insoluble material, mainly fats. The supernatant liquid is adjusted to 50 per cent ethanol and washed with carbon tetrachloride. The CCl_4 phase is rejected and the alcoholic phase evaporated and dissolved in 2 ml. of 50 per cent ethanol.

In our earlier work we had encountered considerable difficulty with lipid material which caused blocking of the partition columns and displacement of the steroid zones. This was overcome by extraction of the interfering material on a reverse

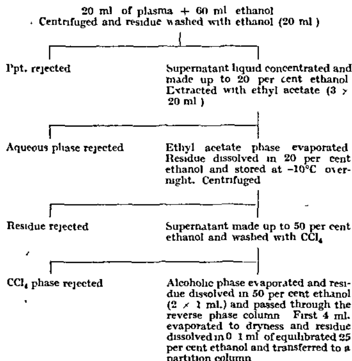


FIG. 1.

phase partition column. When Hyflo Supercel is treated with the vapour phase of a dialkyldichlorosilane it becomes coated with a surface which is essentially made up of alkyl groups (Howard and Martin, 1950). Such material will retain a non-polar liquid, and thus a partition system can be set up in which the non-polar is the stationary phase and a polar liquid can be used as mobile phase. The system used here was light

17-hydroxysteroids based on chromatography on a magnesium silicate-Celite column followed by estimation by a micro Porter-Silber reaction. This reaction is believed to be specific for substances with a 17-hydroxyketol side chain and would therefore estimate only Compounds E and F. Nelson and Samuels (1952) examined their 17-hydroxysteroid fraction by paper chromatography using the method of Zaffaroni, Burton and Keutmann (1950) and concluded that it contained only Compound F. They found an average normal range of 4–10 μg. of 17-hydroxysteroids/100 ml. of blood.

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A and B give polarographic waves typical of both Δ^4-3-keto and 20-keto groups, while 17-hydroxycorticosteroids show only Δ^4-3-keto waves; the ketonic character of the 20-keto

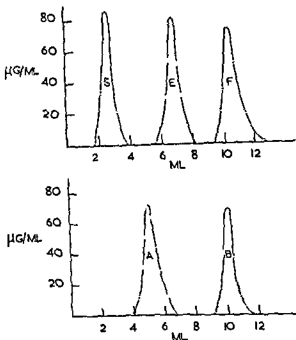


FIG. 2. Partition chromatography of corticosteroids. (above) Compounds S, E, F, in system toluene-aqueous ethanol. (below) Compounds A and B in system toluene-ethylene glycol

group being suppressed under our experimental conditions, presumably by steric or resonance effects.

The lowest amount of a ketosteroid which can be estimated with accuracy by the usual polarographic method is about 10 µg. We have increased the sensitivity of the method 10–20-fold by a new Girard technique suitable for quantities down to about 0·2 µg. For this purpose an inert solvent is required, as the reaction cannot be carried out in anhydrous

petroleum (b.p. 80°–100° C.) as stationary phase and 50 per cent ethanol as mobile phase. The corticosteroids pass directly through the column while the interfering lipids are very efficiently extracted by the very large surface of stationary phase presented. The first 4 ml. of column effluent are collected, evaporated to dryness *in vacuo*, dissolved in 0·1 ml. of equilibrated 25 per cent ethanol and transferred to the first analytical column.

Two partition columns have been used for the separation of the corticosteroids. The column dimensions are 6 mm. diameter by 8 cm. length. In each case the supporting phase is acid-washed Hyflo Supercel. The first column uses 25 per cent ethanol as stationary phase and toluene as mobile phase, *the two phases being thoroughly equilibrated by shaking* before use. The technique of column packing, etc., follows that described earlier from our laboratory (Butt, Morris, Morris and Williams, 1951). The second analytical column uses ethylene glycol as stationary phase and toluene-20 per cent light petroleum (b.p. 80°–100° C.) as mobile phase. The separations possible with these systems are shown in Fig. 2.

In the first system Compounds A and B run practically with the toluene front and are collected in the first 2 ml. Compounds S, E, and F separate as shown (20 μg. of the pure steroids were used for the separations shown in the figure). Samples (1·0 ml.) are collected automatically on a fraction collector. The first fraction from the first analytical column is evaporated to dryness, taken up in 0·1 ml. of ethylene glycol and transferred to the second analytical column (toluene-20 per cent light petroleum-ethylene glycol), which separates Compounds A and B as shown. The individual fractions are freed from solvent *in vacuo* and are then ready for conversion into the Girard hydrazones for polarography.

Estimation of ketosteroids by polarography of the Girard hydrazones was first developed by Hershberg, Wolfe and Fieser (1940). It has been used in our laboratory for several years. It has the particular advantage that it gives a qualitative as well as a quantitative analysis. Thus Compounds

polarography and evaluation of the polarograms has been described earlier (Butt, Morris, Morris and Williams, 1951).

The method described for the determination of corticosteroids in blood appears complex but it is possible to carry out five complete estimations in a week.

Compounds A, B, E and F, in amounts of 5 and 10 μg., added to 20 ml. of plasma can be recovered to better than 80 per cent. The recoveries of mixtures also indicate that during the processing there is no interconversion.

Table I contains some normal values obtained by the method. Compounds A and E were found, as well as the B and F reported by other workers. In no case has Compound

Table I

BLOOD CORTICOIDS IN NORMAL SUBJECTS

No.	*Sex*	*Compound (μg /100 ml plasma)*			
		A	*B*	*E*	*F*
1	M	4 5	8 5	4 0	7·0
2	M	3·5	7 5	3 5	10·5
3	F	5 0	10 5	4 0	7·5
4	F	2 0	4 5	2 5	6·5
5	F	3 0	9 0	4 5	9·0

BLOOD CORTICOIDS IN NORMAL PREGNANCY

No	*Term (weeks)*	*Compound (μg /100 ml plasma)*			
		A	*B*	*E*	*F*
1	full	5·0	18 0	7 5	18 5
2	34	8 5	15 0	9 0	23 5
3	32	4 5	12 5	4 5	15 5
4	34	2 5	7 0	6 5	8 5

S′ been detected even when much larger quantities of blood have been worked up. The amounts of B and F are approximately twice the amounts of A and E. The compounds running in positions A and B show the double wave typical of 3.20-ketosteroids without a 17-hydroxyl group. The com-

acetic acid only. The criteria for this solvent are very rigorous, as it must be a good solvent for steroids and also for the very polar Girard reagent, and should preferably be miscible with water. Since about 100 mg. are used it must contain less than 1 part per million of polarographically reducible impurity. After very many unsuccessful trials, a suitable solvent was found in *tert*-butanol purified by 10 successive extractions with half-saturated KCl solution, drying and distillation. The final reaction mixture is a 0·2 per cent solution of Girard re-

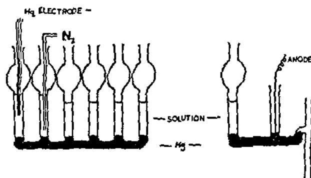


FIG. 3. Micro-polarographic cell for estimation of corticosteroids.

agent T in purified *tert*-butanol containing 2 per cent acetic acid. The steroid fraction is heated in a stoppered tube with 0·05 ml. of this mixture at 100° C. for two minutes and the butanol and acetic acid removed *in vacuo*. The mixture is treated with 0·2 ml. of toluene and 0·2 ml. of the polarographic base solution (0·5 N KCl 1 vol., 0·2 N KOH 2 vol., water 1 vol., adjusted to *p*H 5·6 with glacial acetic acid). The mixture is shaken, allowed to settle, and the aqueous layer transferred to the micro polarographic cell shown in Fig. 3.

The cell is particularly useful for multiple determinations, as the solutions can be put in the separate tubes and degassed with nitrogen while the earlier ones are being polarographed. The mercury in the tubes is maintained at a constant level during polarography by the overflow tube. The technique of

muscularly, and a second blood sample taken one-and-a-half hours later. In cases 1 to 3 the subjects were allowed to move about during the one-and-a-half hours interval, while cases 4 and 5 were resting. In all cases there was a marked increase, particularly for Compounds B and F, but the rise appears to be more marked in the resting cases. However much more experimental data will be necessary to establish this point.

We have also studied the blood of the rat after ACTH administration and have confirmed Bush's finding that in this species the steroid formed by the adrenals is almost entirely Compound B with a very little F.

In conclusion, we hope this technique will be of value for studying adrenal physiology and biochemistry both in man and in experimental animals.

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DISCUSSION

PINCUS I would like to ask Dr. Morris about the distribution of these substances between the red cells and the plasma. Very preliminary work which we did with beef blood suggested there might be a distribution there

MORRIS We have no experience yet with blood,* it's one of the things we're proposing to do. I should say that Dr Samuels's figures on blood and ours on plasma agree pretty well, and if there were a

*A comparison between corticosteroids in blood and plasma on the same blood specimen has now shown that the corticosteroids are present solely in the plasma.

pounds in positions E and F, on the contrary, give no 20-keto wave.

The presence of Compound E was rather surprising as it does not appear to have been observed before in human blood. The compound running in this position has a Δ^4-3-keto group, reduces blue tetrazolium salt and gives the fluorescence when heated with alkali on paper which Bush has described as diagnostic for an $\alpha\beta$-unsaturated ketone with at least two rings.

A preliminary experiment on one case using the quantitative blue tetrazolium method of Mader and Buck (1952) indicates that the Δ^4-3-keto and reducing groups are present in a 1:1 ratio. In addition, the absence of a 20-keto wave suggests that the compound is a 17-hydroxyketol. We hope to settle this question of identity later on.

The table also shows the values obtained in pregnancies. In agreement with earlier findings with urinary reducing and formaldehydogenic steroids, there is an increase of about 100 per cent in all corticosteroids measured.

Table II illustrates the effect of pituitary adrenocorticotrophin on the blood corticosteroids. In all cases a blood sample was taken as control, 25 i.u. of ACTH injected intra-

Table II

BLOOD CORTICOIDS IN NORMAL AND ACTH-TREATED MALES

Case	*Treatment ACTH*	*Compound (µg /100 ml plasma)*			
		A	*B*	*E*	*F*
1	None	2 0	5 5	5 5	5 0
	25 i u.	3 0	19 5	8 5	20 5
2	None	5 0	10 0	4 5	8·0
	25 i u	9 0	18 5	7 5	20 0
3	None	3 5	9 0	5·0	9·0
	25 i u	10 5	16 5	7·5	16·0
4 (resting)	None	7 5	10 0	5·0	12·5
	25 i u	14 5	19 0	40 0	56·5
5 (resting)	None	4·0	7 5	5·0	8·0
	25 i u.	17 5	27·0	33·5	42 5

MORRIS Not of B?

BUSH: Not of B

MORRIS: I think we must be very sure that the method is effective. You see we were recovering of the order of 85 per cent of B We shall have to be quite sure, interpreting the difference between 100 and 85 per cent, that the method is in fact recovering quantitatively

BUSH: I have determined recovery values for B from a whole gland extract, and found that the ratio of F to B in the gland extract after this extraction procedure was the same as in the original gland extract. So that it doesn't seem that the recovery can be all that much less—it might be 70 per cent instead of 85 per cent This was my sole source of compound B until very recently

MORRIS I think it's very hard to say unless you've done the actual experiment, because I think the nature of the extract will have something to do with it, and of course the actual amounts you're dealing with These estimations of ours, with these quantities have an error of at worst ± 10 per cent—probably much better than that

BUSH. I'm not saying that you don't get B off your columns, but I'm wondering whether you've never found a blood in which you got a ratio of F to B of 5

MORRIS No. We have, however had one case with a high B an even lower ratio F/B

SAMUELS Could it be that the compound which is running in that region might not be B but some more reduced compound whose polarity is offset by other differences?

MORRIS But the substance which runs in the B position is a Δ^{4}-3 20-ketosteroid I may say that the polarographic waves obtained after the column separation are almost indistinguishable from those given by the pure compound.

SAMUELS We found that after exercise there is a significant rise in the level of these substances in the blood and also in the urine Also the excretion in the urine is twice as high during the day as at night In other words, in the intact individual it looks as though there is increased total corticosteroids with exercise, yet after ACTH, Dr Morris's evidence would indicate that the output is greater when resting This could be associated on the basis that in the individual who exercises the increased output of ACTH more than compensates for the disappearance of steroid. There is some other evidence that points that way in some of Dr Sayers's original data It raises a very interesting question.

MORRIS With respect to the difference in ACTH effect on resting and mobile subjects, we don't feel very confident about it yet because we

retention of steroids on any elements in the blood this would not be the case; there obviously isn't a great deal, allowing for the hæmatocrit.

BUSH: Dr. Bibile, working with Dr. Marthe Vogt, investigated this question of cell/plasma distribution in adrenal venous blood, and found that if he separated the cells and plasma immediately there was no detectable eosinopenic activity in the red cells; it was all in the plasma. But he found that if you left the blood standing before centrifuging, for a matter of five hours I think the figure was, you could get something like a 50:50 distribution between the cells and the plasma.

HECHTER: This work of Dr. Bibile seems very important. One might assume that in adrenal venous samples, where the corticosteroid presumably enters the plasma compartment first, practically all of the corticosteroid might be expected in the plasma. The adrenal venous blood then mixes with the systemic circulation and then, if Dr. Bibile is right, in time corticosteroid could enter the red cells This raises the possibility that one might get entirely different results with respect to corticosteroid distribution between red blood cells and plasma, depending on whether one uses adrenal venous or peripheral blood samples.

SAMUELS: I think, however, that Dr. Nelson and his group did check the same sample of blood, whole blood and plasma, and within the limits of the method (remembering that this is the Porter-Silber reaction) found no significant amount in cells And for this reason they used plasma after that because it eliminated the question of the large amount of protein from the cells

MORRIS Yes, that was our main reason.

BUSH: I would like to comment on the large amounts of corticosterone that Dr. Morris has found We've done a small series of determinations (using just the paper method, putting the extracts through a much simpler process) on the blood of women in normal pregnancy [illegible] In

[illegible]

be roughly 1:1 with the two compounds. The only case we have in which corticosterone was very pronounced was in a case of pregnancy toxæmia which was very interesting. About 12 hours before delivery she had a total content of Δ^{4}-3-ketosteroids of 80 μg per 100 ml of blood, and with a completely reversed ratio—1 part of 17-hydroxy- [illegible] parts of corticosterone. Approximately 20 hours

[illegible]

way through

BUSH: We've only carried out recoveries of F and E on blood.

kidney, muscle, and other tissues to yield sizeable amounts of metabolites. Used in this fashion the perfusion technique serves two functions: it is a biochemical tool for the evaluation of the *enzymatic capacities* of the perfused organ; and a *manufacturing plant* for the production of transformation products. It is *not* necessarily a system for studying organ function as it exists *in situ* under physiological conditions.

In view of the dominant rôle of the liver in diverse metabolic processes, including the metabolism of steroids, studies were undertaken some two years ago to investigate the influence of perfused hepatic tissue upon corticosteroid transformations. In this report, I should like to present some of our preliminary findings.

In the initial stages of this work, search was made for a convenient perfusion system adequate to demonstrate hepatic enzymatic activity upon corticosteroids Using deoxycorticosterone (DOC) as a model, we were soon able to demonstrate that upon perfusing DOC in a variety of media (homologous blood, heterologous beef blood, or Ringer's solution containing 6 per cent polyvinyl pyrollidone or gelatin) through rat or rabbit liver, that the added steroid disappeared from the system when the perfusate was recycled 100–125 times through the organ. Similar results were obtained whether the livers were perfused through the portal vein, or backwards through the hepatic vein, reversing the normal course of liver circulation. While the qualitative picture of "corticosteroid utilization" under these diverse conditions was similar, these results should not be taken to indicate that there may not be qualitative or quantitative differences in the course of corticosteroid metabolism between rat and rabbit liver, or between different perfusion conditions.

Having demonstrated that perfused rat and rabbit liver under a wide variety of conditions are active biochemical preparations with respect to DOC, we decided to adopt an arbitrary set of conditions and study the metabolism of corticosteroids systematically. Having obtained basic information for one set of conditions, it would be possible to study the

CORTICOSTEROID METABOLISM IN ISOLATED PERFUSED RAT LIVERS*†

OSCAR HECHTER

WHILE there is no crucial evidence for or against the view, some endocrinologists concerned with hormone action at a molecular level have privately speculated whether the hormones, particularly the adrenocorticosteroids, may not act in themselves, but require metabolic conversion to an "active intermediary" before they exert effects upon target organs. With the dramatic and unexpected demonstration that cortisone, in large doses, exhibits a multiplicity of effects in a variety of physiological and pathological processes, it seemed to us that in cortisone we might have a tool for testing this hypothesis. Rejecting indirect experimentation, we felt that a long-term project should be undertaken to systematically chart the course of cortisone metabolism in various organs, and, as metabolites were isolated and chemically characterized, to assess their biological activity in a variety of tests (not only the glycogenic and electrolyte bioassays usually employed for evaluating corticosteroid activity), first in animals and then in man.

To obtain the large amount of transformation products necessary for this type of work, it appeared likely that the perfusion technique (Hechter *et al.*, 1951), which had already proved useful in evaluating corticosteroidogenesis in perfused bovine glands, when applied to other organs, using cortisone as the substrate, would provide a profitable approach to this problem. Once conditions of reaction had been established, gram quantities of cortisone could be reacted with liver,

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†The work reported was done in collaboration with Dr. M. M. Solomon, I. A. Macchi, E. Caspi and M Feinstein

Table I.
RECOVERY OF RADIOACTIVITY FROM LIVER PERFUSATES (CHARCOAL METHOD)*

Sample	ml	Total Counts		Per cent Recovered
		Introduced	Found	
Control	50	10,400	9,921	96
1 cycle perfusion	50	,,	6,146	59
10 ,, ,,	50	,,	4,884	47
50 ,, ,,	50	,,	5,019	48

Total Counts lost from perfusate =	41,064
Counts in Liver Fractions	
70 per cent EtOH	2,090
Pentane	0
Counts in Pump Washings	800
	2,890

*^{14}C-labelled DOC of activity 2,080 counts per mg per min. perfused at concentration of 10 mg per 100 ml blood

no case were significant counts obtained by these treatments.

Using the chloroform-ether method of Nelson (1952) or the ethyl acetate procedure developed by Andre Meyer at the Worcester Foundation, we have been able to recover the missing activity, not isolated by the charcoal procedure. Table II presents data on another liver perfusion experiment with ^{14}C-labelled DOC, where samples were removed after 1, 5 and 10 cycles of the perfusion media through the liver.

Table II
COMPARATIVE RECOVERY OF RADIOACTIVITY FROM LIVER PERFUSATES WITH THE CHARCOAL AND SOLVENT EXTRACTION METHODS

Sample	Per cent Recovery	
	Charcoal	Ether-chloroform (4 1)
Control	92	96
1 cycle perfusion	61	100
5 ,, ,,	59	96
10 ,, ,,	61	79

influence of variables such as species, perfusion media, etc., in terms of a standard. The conditions selected were convenient but not necessarily physiological. Rat livers were chosen, and they were perfused through the hepatic vein with citrated bovine blood containing 10 mg. corticosteroid per 100 ml.

Our evaluation of "corticosteroid utilization" had involved our previously described charcoal extraction procedure (Hechter *et al.*, 1950) for the extraction of corticosteroids from liver perfusates. While this method leads to good recovery of added corticosteroid hormones (such as DOC, corticosterone, Kendall's F, Reichstein's S) from blood, we had no information whether the transformation products formed by hepatic enzymatic systems would also be extractable from blood by charcoal, and eluted from it without modification. Accordingly, to check our extraction procedure, 40 mg. ^{14}C-labelled DOC (equally labelled at C-3 and C-21; radioactivity of 2,080 counts per minute per mg.) was added to 400 ml. of blood and perfused through a rat liver for 1, 10 and 50 cycles. At each time interval a 100 ml. sample was removed. The perfusates, as well as a control sample, were treated with charcoal, the steroids eluted by soxhlet treatment with methylene chloride, and the residues counted. The perfused liver was extracted for lipids by acetone and chloroform treatment, and the lipid residue partitioned between pentane and 70 per cent aqueous ethanol. The results of the recovery of radioactivity are illustrated in Table I. It will be seen: (1) that there is a complete recovery of the added radioactivity in the control sample, demonstrating that the charcoal method employed is adequate for added DOC; (2) starting with 1 cycle perfusion and continuing through 50 cycles perfusion, a loss of 40–50 per cent of the radioactivity is observed. Only a small percentage of the counts lost can be accounted for in terms of liver accumulation or adsorption on the walls of the apparatus. To test the possibility that in liver-perfused samples, products were adsorbed on charcoal but not eluted by methylene chloride, the charcoal was exhaustively extracted with methanol, ether, pentane and pyridine, but in

The experimental design in most cases was as follows: 600 ml. of citrated bovine blood was prepared containing 60 mg. steroid (added as an ethanolic solution dropwise to rapidly swirling blood to give a final concentration of 1 per cent ethanol). After approximately thirty minutes (for complete mixing) 50 ml. was removed as a control sample (No. 1), and 400 ml. was added to the perfusion apparatus A freshly prepared rat liver was inserted and perfusion initiated. The remaining 150 ml. of blood was transferred to another pump, not containing a liver, and circulated for the entire duration of the liver perfusion experiment to determine the influence of blood incubation alone upon the added steroid (Control No. 2). Knowing the volume, and measuring the blood flow, it is possible to calculate the number of circulations of perfusate through the organ. At 1, 5 and 10 cycles. 50 ml. samples were removed from the venous outflow of liver.

These perfusate samples, as well as the two control samples, were then extracted with ether-chloroform (4 1) and a variety of tests were carried out upon the residues. Fifty per cent of the material was subjected to paper chromatography (Zaffaroni *et al*, 1950) to determine the absolute disappearance of the added corticosteroid and the appearance of new compounds as evidenced by spot tests. The remaining material without further purification was utilized to measure changes in various functional groups in the corticosteroid molecule. For the ketol side chain, we employed the blue-tetrazolium (B.T) method of Chen and Tewell (1951); for the detection of possible glycols as well as ketols, the formaldehydogenic steroids (FS) (Daughaday *et al.*, 1948) were measured. To evaluate changes in the $\alpha\beta$-unsaturated 3-ketone grouping, we measured U.V absorption at 240 mμ,, and finally, to check the production of possible 17-ketosteroids arising from the degradation of the corticosteroid side chain, we measured the Zimmermann chromogen (Z) at 515 mμ, on the basis that if true 17-ketosteroids were to be formed one should expect a rise in the titre. In all of the latter determinations, the values obtained were corrected

In this case, one portion of the perfusate was extracted with ether-$CHCl_3$ (4:1), while another was extracted by our charcoal method. It will be seen that although only 60 per cent of the radioactivity is isolated by our charcoal method after 1 cycle through the liver, it is possible to quantitatively extract all of the radioactivity from 1 and 5 cycle liver perfusates with ether-chloroform. At 10 cycles, ether-chloroform recovers only 79 per cent of the added counts. Time does not permit a detailed description of experiments designed to elucidate the differences between the charcoal method and the solvent extraction procedures. Suffice it to say that, while all DOC transformation products appear to be adsorbed upon the charcoal (since subsequent solvent extraction with ether-chloroform does not yield radioactivity), it seems likely that some transformation products undergo modification on charcoal, so that the labelled carbon at C-21 or C-3 is lost.

Fractionation of perfusates obtained with ^{14}C-labelled DOC, indicates that the radioactivity is not extractable to any significant extent from chloroform by either 0·1 N H_2SO_4 or by 4 per cent $NaHCO_3$. It would thus appear that the bulk of the products derived from DOC perfusion represent neutral materials.

Having suitable methods for the extraction of DOC transformation products from liver perfusates, and knowing that this corticosteroid does not accumulate in any significant degree in the perfused liver, we have perfused four corticosteroid hormones under the same arbitrarily fixed conditions. In addition to DOC and cortisone, we have perfused 17-hydroxy-11-deoxycorticosterone (Reichstein's S) and 17-hydroxycorticosterone (Kendall's F), at a level of 10 mg. steroid per 100 ml blood at non-pulsatile perfusion pressures ranging from 40–48 mm. Hg. Large rats were employed in these studies, and their livers weighed 10·8 to 23·2 g. at the end of the experiment. The degree of œdema in these perfused livers is not large (less than 5 per cent of the total weight), and the hepatic blood flow under our conditions ranged from 2·4 to 4·5 ml. per min. per g. liver.

for hepatic transformation of all of these corticosteroid hormones is "utilized" to approximately the same degree. Further experiments would be necessary to determine whether the differences noted between individual steroids in this small series are really significant.

The degree of transformation achieved can best be appreciated if we were to extrapolate these values to human livers, not as an example of what one should expect under physiological conditions, but only for illustrative purposes. Assuming a liver weight of 1,500 g., this would represent the conversion of about 150 mg. of cortisone (or related steroid) per minute of circulation. This is truly an extraordinary capacity for the conversion of corticosteroids. It is only fair to add that this very high rate may be dependent upon the artificially high levels of corticosteroid added to the blood. Lower levels of "corticosteroid utilization" may be expected if the concentration of steroid substrate is lowered toward levels which exist under physiological conditions.

Nature of the Transformation Products Produced

Table V illustrates the results of a typical experiment with cortisone, but the general picture holds for the other steroids tested as well. For each determination, the particular func-

Table V

DISAPPEARANCE OF VARIOUS FUNCTIONAL GROUPS (RELATIVE TO THE ADDED CORTISONE) INDUCED BY RAT LIVER PERFUSION

Sample	*Per cent recovery of added steroid as*				
	Cortisone	*B T*	*F S*	*U V*	*K S*
Control No 1	95	96	92	88	86
Control No 2	93	97	88	86	88
Cycle 1	66	87	89	75	75
3	54	68	72	57	52
5	44	66	70	48	36
10	20	64	66	31	25

B T = blue tetrazolium test
F S = formaldehydogenic steroids
U V = absorption at 240 mμ.
K S = 17 ketosteroids

from a blank obtained by extracting a sample of blood to which no corticosteroid had been added.

Rate of Disappearance of Corticosteroids

By measuring the added corticosteroid present in the blood before and after the first hepatic circulation (the corticosteroid [illegible]

a level of 10 mg. corticosteroid per 100 ml. blood. In Table III, the results of three experiments with cortisone are presented. It will be seen that 1 g. rat liver perfused under

Table III

RATE OF CORTISONE UTILIZATION BY PERFUSED RAT LIVER*

Expt	*Cortisone lost*	*Liver wt*	*Perfusion time*	*Rate*†
	mg	g	min	
1	3·76	17	1 80	123
2	3 42	20	1 86	92
3	2·90	23	1 66	78

*Perfused at a cortisone concentration of 10 mg per cent
†μg lost per g liver (wet wt) per min perfusion

these conditions can transform about 100 μg. of cortisone per minute. Similar results are shown for DOC, Reichstein's S, and Kendall's F in Table IV. It will be seen that the capacity

Table IV

RATE OF UTILIZATION OF VARIOUS CORTICOSTEROID HORMONES BY PERFUSED RAT LIVER

Corticosteroid	*No. Expt.*	*Rate**
Cortisone	3	100
17 OH-corticosterone	3	123
DOC	3	120
17 OH-DOC	3	130

*μg lost per g liver per minute of perfusion.

the starting material. The loss of the ketol side chain in the remaining products by conversion to glycols is excluded, since the F.S. values, which measure glycols as well, are similar to the B.T. values. No evidence for a significant conversion of cortisone (or any of the corticosteroids tested) to 17-ketosteroids, was obtained, as indicated both by the steady decrease in Zimmermann chromogen at 515 mμ, and by carrying out the Zimmermann reaction on paper chromatograms Accordingly, the loss of ketol side chain cannot be explained in terms of this mechanism.

(c) Disappearance of the Conjugated Unsaturated System

In all the corticosteroids tested, the Δ^4-3-ketosteroid grouping seems to be more rapidly removed than the ketol side chain, under our conditions of liver perfusion. The U V. values shown in Table V for the experiment with cortisone most probably overestimate the actual values, for our paper chromatograms reveal that practically none of the major transformation products formed gives an orange spot with the dinitrophenyhydrazine reagent, which is sensitive to and characteristic for Δ^4-3-ketosteroids. This hydrazine reagent reacts with 3-ketosteroids to give yellow spots, and while our paper chromatograms reveal a few spots of this type, it seems probable that the bulk of the transformation products no longer retain the 3-ketone grouping.

Having this basic information, we have undertaken large scale perfusion of DOC and cortisone to attempt to identify the metabolic products formed. At the present time we are engaged in fractionating 3 g. of cortisone transformation products by a combination of silica gel and paper chromatography. To date, the most striking feature of our results with DOC and cortisone is that a galaxy of products is formed, a conservative estimate would be a minimum of 20 metabolic products, most of which contain neither 3-ketone nor the Δ^4-3-ketosteroid grouping. In confirmation of Schneider and Horstmann (1951) we find *allo*pregnane-3β:

tional groups, measured in terms of cortisone equivalents, are expressed as percentage recovery of the added steroid.

(a) Influence of Blood Incubation, in the Absence of Liver

Following incubation of cortisone (as well as the other corticosteroids tested) in blood at 38° for 90–120 minutes (by circulating the mixture through the apparatus) the bulk of the added steroid is recovered unchanged. In each case, however, there is evidence by paper chromatography for the development of trace amounts of new steroid ketols, more polar than the corticosteroid substrate. This, however, should not necessarily be interpreted to indicate the presence of specific factors in blood, for similar results were obtained when DOC (the only substance so tested) was incubated at 38° with Ringer's-polyvinylpyrollidone. Remembering that Wintersteiner and Bergstrom (1941) found that cholesterol in colloidal solution in the presence of oxygen, undergoes autooxidation at C-7 (to form 7-hydroxy- and 7-keto-cholesterol), presumably because that position is activated by the double bond at the 5:6 position, one wonders whether the products formed by circulating corticosteroids in oxygenated blood may not give rise to 6-hydroxy or 6-keto- corticosteroid derivatives. In passing, it may be of interest to mention that one of the products formed from DOC under these incubation conditions without liver gives a cortisone-like, blue coloration with iodine, in contrast to the yellow brown spots usually produced by Δ^4-3-ketosteroids (Zaffaroni *et al.*, 1950).

(b) Disappearance of the Ketol Side Chain

Although the added cortisone disappears from the medium as liver perfusion is continued, the perfusate still contains steroid ketol, as measured by B.T., to the extent of 40–50 per cent of the original value. This indication that some of the transformation products formed retain the ketol side chain is fully confirmed by paper chromatography, which reveals a variety of new ketol spots, most of which are more polar than

in livers, not only of the rat but of a variety of species, before it will be possible to speak with any real understanding of the pathways of corticosteroid metabolism in liver. By this time next year we hope to be in a position where we can speak definitely about the entire spectrum of hepatic transformation products derived from cortisone, and perhaps some estimate of their biological activity.

When this work was begun, the local effectiveness of cortisone in certain conditions (notably in certain skin and eye conditions) led some to extrapolate to a point where all cortisone action was explicable on a "local" basis, rendering unnecessary the postulation of an active metabolite formed in a distant organ such as liver During this period our faith in the "active metabolite" view was maintained by our finding that deoxycorticosterone and cortisone perfused in a variety of media, through either rabbit or rat livers, rapidly disappeared from the system, in agreement with studies by others using liver slices (Schneider and Horstman, 1951, Paschkis *et al*, 1951; Louchart and Jailer, 1952) that hepatic tissue possesses active systems for the transformation of added cortisone. These *in vitro* findings assumed for us major significance when it was reported that cortisone was only slightly less active by mouth than by parenteral injection, in terms of anti-arthritic activity in man and thymolytic activity in mice (Freyberg *et al.*, 1950, Boland and Headley, 1951; Patterson *et al.*, 1951, deAndino *et al.*, 1951) Since oral administration of steroids, which is generally believed to involve direct passage of the steroid through the liver, did not lead to marked loss of cortisone activity as evaluated by these tests, these findings seemed to us to be strongly suggestive that one or more of the cortisone transformation products formed in liver might be an effective anti-arthritic agent.

I am aware that upon incubating cortisone for many hours with liver slices there is a loss of glycogenic activity (Paschkis *et al.*, 1951; Louchart and Jailer, 1952). I am also aware that the work of Schneider and Horstmann (1951), as well as our own, points to a rapid reduction of the conjugated unsaturated

21-diol-20-one as a metabolite of DOC, but in addition we find several DOC metabolites which give a positive blue-tetrazolium test and a cortisone-like blue coloration with iodine. One of these, of polarity somewhat similar to cortisone, has been purified and subjected to infra-red analysis, which has clearly revealed that it is not cortisone. Although the structure of this compound is still unknown, one wonders whether *this* liver metabolite of DOC may not explain the conversion of DOC to cortisone by liver slices described by Seneca and co-workers (1950).

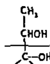
What is the nature of these metabolites of cortisone which retain the ketol side chain? Theoretically, 4 tetrahydro derivatives are possible from cortisone, and to date we have evidence for one; *allo*pregnane-3α-17α:21-triol-11:20-dione appears to be a major metabolic product. Two dihydro derivatives of cortisone seem likely, and we have evidence for both as minor products. If the liver possesses a system for the reduction of the 11-keto group to 11β-hydroxyl, as seems possible, we may expect corresponding tetrahydro and dihydro derivatives of Kendall's F.

While we have some notions as to the character of the metabolites retaining the ketol side chain, we have no information as yet about the metabolic products in which the ketol side chain has been lost. Since both DOC and 11-dehydrocorticosterone have been reported in humans to be metabolized to pregnanediol (Hoffman *et al.*, 1943) and 11-ketopregnanediol (Mason, 1950) respectively, it is possible that products with the C-21 carbinol reduced to a C-21 methyl and/or the C-20 ketone reduced to a C-20 hydroxyl may be present in our mixture. On the other hand, it is equally possible that the cortisone side chain is degraded to form 17-ketosteroids, which then in turn are rapidly converted to other products by liver enzymes, at a rate sufficient to prevent the accumulation of 17-ketosteroids such as 11-keto- or 11β-hydroxy-androsterone or -etiocholanolone. In either event, speculation is not profitable. Further work is necessary, systematic isolation and characterization of the products of corticosteroid metabolism

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DISCUSSION

MARRIAN: I was interested in your suggestion that you might get corticosteroid metabolites of this type, and in that connection, I think



it would be rather interesting for you to try out on your perfusates a

steroids.

HECHTER: I should like to show another slide (Table VI) Let us

the rate of disappearance of steroid in slices tends to approach the perfusion data as the period of slice incubation is decreased The last part of the table represents data from Chernick and Chaikoff from experiments in which liver slices were incubated with uniformly labelled glucose, and the rate of glucose oxidation calculated by measuring radioactive CO_2 One can calculate the amount of hydrogen transferred from glucose to form the water in the oxidation to be 8 4 micro-

system in corticosteroids, regarded as essential for activity in the corticosteroid series. But I would remind you of recent demonstrations by Grundy, Simpson and Tait (1952) which strongly suggest that the mineralocorticoid activity of beef adrenal extract is largely accounted for by a steroid ketol many times more active than DOC, which does not appear to possess the Δ^4-3-ketosteroid grouping. This finding, if established definitively, would liberate us from the dictum that biological activity in the corticosteroid series requires the $\alpha\beta$-unsaturated ketone grouping in Ring *A*.

In the light of these new developments, all compounds implicated in the metabolism of cortisone and related compounds deserve full investigation of their biological activity, not in a single bioassay procedure, but over the full spectrum of the biological activity which corticosteroids exhibit.

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the levels rise, the amount removed increases (as one might anticipate from the mass action effect) so that at high levels the removals are about 50 per cent on one circulation. This sounds like an extremely rapid removal, but removals of testosterone and progesterone are higher, so this really isn't out of order. And of course one of the effects of oral admini-

more marked effect than if you had a lower level over a longer period of time, because once in the cells the hormone becomes bound with the enzymes and its effect is prolonged The fact that cortisone is not directly active leads to the conclusion that it is probably reduced to Compound F. But I don't believe that we have to assume that some of the other compounds are activated to get the effects which we note It certainly should be investigated, but I don't think it's an essential assumption

DJERASSI I want to mention one brief point The two compounds that Dr Hechter suspects were formed in his liver perfusion were Reichstein's Compound C, which is an *allo* (5α) steroid, and dihydro-*allo*cortisone, not the usual dihydro E As I mentioned in the discussion after Dr Pincus's paper, the corticoids you isolate from urine are

necessarily hold true.

find in humans and monkeys.

HECHTER. I feel that our liver perfusion studies need much amplification in many directions One feature of

Table VI

CORTICOSTEROIDS AS HYDROGEN ACCEPTORS

Corticosteroid + $2H_2$ ⟶ reduced products

Reference	*Conditions*	*Period*	*Rate of Disappearance*		H_2 *uptake*
		min	*µg/hr./g*	*µM*	*µM/hr/g*
This report	perfused rat liver glucose-citrate cortisone 0 27 µM per ml.	1–2	6600	18 5	37·0
Schneider and Horstmann* (1951)	rat liver slices glucose DOC 1 5 µM per ml.	15 60 180	2672 976 444	8 0 3·0 1 3	16 0 6 0 2 6

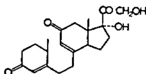
$C_6H_{12}O_6 + 6O_2$ ⟶ $6CO_2 + 6H_2O$

Reference	*Conditions*	*Period*	*Rate* ^{14}*C glucose*, $^{14}CO_2$		H_2 *removed*
		Min	*µg/hr/g*	*µM*	*µM/hr/g*
Chernick and Chaikoff† (1951)	rat liver slices ^{14}C-glucose no steroid	180	257	1·4	8·4

**J biol Chem*, 1951, 191, 827
†*J biol Chem*, 1951, 188, 389

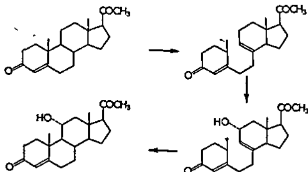
extending
ein-
of
ion,
, as

evidence that cholesterol contains a companion substance, present to the extent of about 0 5 per cent, which from the analytical data we have at the present time seems to be related, not to cholesterol, but more to a D-type vitamin. It may be what one can call a *seco*-cholesterol, having all the structural features of cholesterol itself, but opened up between rings *A* and *B*. If something like this can accompany cholesterol and can remain undetected for many years, you can see the possibility that for every hormone there is a *seco* hormone (for example, *seco*-cortisone) and that such a thing is the active biological agent Now



this is rather attractive because you'd only have to postulate that such a substance is present in very minute amount and that it is formed only in contact with an enzyme system. If you look for this thing,

biological oxidation at C-11 may take the path of formation of a *seco* derivative, allylic oxidation at C-11, and ring closure



sizeable fraction of metabolites from the administered steroid is unrecognized. Although, at the present time, most of the products from the liver perfusion are unknown, I think that we have an opportunity to pick up the missing metabolites using this technique.

Whether this mixture will represent a mixture of biologically active and inactive substances, I ... I ...
variety of *in vitro* systems ...
action might be expected ...
would very much like to ...
mixture possessing high biological activity. However, I agree with Dr. Samuels that at this time my view is just speculation. I'm hopeful, however, that there might be something to this notion, in which case ...

marked effect.

SAMUELS: In both perfusion and tissue studies, I think there's one thing that we have to recognize as different from what happens in the normal intact animal, and that is that we do not necessarily supply the components of the conjugating systems which normally play such an important rôle We certainly see that in our testosterone-liver studies, where we get conversions to compounds more reduced than androsterone, and these are free; but in the intact animal a much larger proportion stops at the androsterone-ætiocholanolone stage, largely because conjugation can take place more readily and all the components for formation of glucuronic acid are there.

PINCUS: In liver clearance studies, would you not be dealing with ...
... here.
... in the disappearance of ...

SAMUELS: It could well be. I think conjugation influences the stage to which the conversion goes. I don't think you get conjugates of F or E; these are first reduced in Ring *A*. Apparently the 3α-hydroxy compounds, and perhaps the 3β also, are very readily conjugated, and as such drained off in the kidney

HECHTER: I might add that we have searched, especially in the initial stages of our work, for conjugated steroids. We have not succeeded in demonstrating any significant conjugation. The evidence that conjugated steroids are important in species other than man is ... I know of no unequivocal evidence that steroids are ...
... evidence isn't by any means ...
... tainly it excretes most of it via ...
... er had some evidence that the ...

SUBJECT INDEX

There has been no attempt to index all the compounds mentioned in the papers and discussions, but only those of considerable physiological interest. Compounds have generally been listed under their trivial names, and [illegible]

AUTHOR INDEX

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